IMPACT OF GAMETE PRODUCTION ON BREEDING SYSTEMS AND POPULATION STRUCTURE OF HYBRIDOGENETIC FROGS OF THE *PELOPHYLAX ESCULENTUS* COMPLEX: THE EVOLUTIONARY POTENTIAL OF INTERSPECIFIC HYBRIDIZATION

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« Le biologiste passe, la grenouille reste »

J. Rostand

Illustration : Water frog couple, from August Rösel von Rosenhof, in *Historia naturalis Ranarum nostratium*, 1758

Contents

Summary

The European water frog *Pelophylax esculentus* (genome LR) is a natural hybrid between *P. lessonae* (LL) and *P. ridibundus* (RR). It presents a peculiar quasi-sexual reproductive mode known as hybridogenesis: the hybrid excludes one of the parental species' genomes at a pre-meiotic stage of gametogenesis, thus producing gametes containing clonal copies of the other parental genome. By mating with the parental species whose genome has been excluded it re-establishes hybridity at each generation.

Moreover, because of its hybrid nature and resulting problems of chromosome pairing at gametogenesis, *P. esculentus* also produces diploid gametes from time to time. These gametes often lead to the generation of triploid frogs which will allow, under certain ecological conditions, the establishment of all hybrid populations which are maintained without the genetic contribution of either parental species. Over the past decade, such populations have been well studied in the north-western part of Europe, but the presence of triploid water frogs has also been reported for various areas in Central Europe. However, for those localities details on the breeding system, i.e. the genetic contribution of the various frog types, are usually lacking. The major goals of this thesis were to (a) investigate the Central European populations more closely, (b) to compare the breeding systems there with that in all-hybrid populations from Northern Europe and (c) find out whether triploid water frogs in different areas are of mono- or polyphyletic origin.

In chapter one I used microsatellite DNA analyses and crossing experiments to compare five populations (one in Poland, two in Germany and two in Slovakia) presenting different population structures. Indices of heterozygosity and of genetic differentiation allowed to depict the genetic interactions between the different type of frogs (LL, LLR, LR, LRR and RR). I was then able to define and differentiate the breeding systems occurring in each of them and to propose an evolutionary scenario for the appearance and maintenance of the all-hybrid populations.

Chapter two presents a collaborative study with Alexandra Hoffmann. Here we enlarged our survey to populations distributed all over Europe and used microsatellite DNA and mitochondrial DNA (mtDNA) analyses to find patterns of genetic structure among different breeding system types. We found that genetic diversity among hybrid populations is influenced by geographic location (latitude, longitude) and by the proportions of parental genotypes in the hybrid population. Furthermore, we identified genetic clusters from both microsatellites and mtDNA, which indicate that there are at least two separate polyploid hybrid clades existing today: one in Northern and East-Central Europe and one in Eastern Europe (Eastern Ukraine).

In chapter three I focused on eight Czech and Slovak populations. Using microsatellite DNA analyses, flow cytometry and crossing experiments I was able to enlighten and describe a new breeding system type of hybrid water frog populations, the “modified LE-system”. It is characterized by a triploid lineage consisting of males only.

Chapter four takes a more ecological approach where I was looking for fitness differences in larval life history traits between the three hybrid types (diploid LR and two type of triploids LLR and LRR) and their parental species (LL and RR) when raised under two temperature regimes. Diploid and triploid hybrids performed better than their parental species under colder conditions. This finding helps to explain why all hybrid populations dominate in the colder northern part of the species distribution.

Chapter five also presents a collaborative study with colleagues from Poland. A detailed microsatellite DNA analysis of 18 loci revealed the origin of an unexpected pentaploid froglet offspring obtained by artificial crosses. Using the dosage effect of seven microsatellite loci I was able to demonstrate that the pentaploid froglet was the result of the fusion of a haploid L sperm with a tetraploid egg containing two times the entire genome of the hybrid mother (LLRR egg). This study illustrates a practical application of the DNA microsatellite dosage effect which allows unraveling the ploidy level and the number of copies of the two specific genomes, *P. lessonae* and *P. ridibundus,* in the hybrids.

In conclusion, this study (a) allows a better understanding of the diversity and complexity of water frog breeding systems containing triploid individuals, (b) demonstrates the multiple origins of triploids from different populations and (c) proposes an evolutionary scenario for the origin and maintenance of all hybrid populations. I argue that such populations represent significant evolutionary units which deserve attention of biologists but also the care of decision-makers in conservation policies.

Zusammenfassung

Der Teichfrosch *Pelophylax esculentus* (Genom LR) ist ein natürlicher Hybrid zwischen *P. lessonae* (LL) und *P. ridibundus* (RR). Er entsteht durch Hybridogenese, eine seltene hemiklonale Fortpflanzungsform: Der Hybrid schliesst während der Gametogenese eines der beiden Elterngenome prä-meiotisch aus und produziert so Gameten, die klonale Kopien des anderen Elterngenoms enthalten. Durch die Paarung mit derjenigen Elternart, deren Genom in der Meiose ausgeschlossen wurde, wird die Hybridität für jede Generation neu hergestellt.

Wie häufig bei Hybriden, treten vermutlich auch bei *P. esculentus* Probleme in der Gametogenese bei der Chromosomenpaarung auf, so dass gelegentlich diploide Gameten produziert werden, die bei Verschmelzung mit haploiden Gameten triploide Nachkommen ergeben. Unter gewissen ökologischen Bedingungen können reine Hybridpopulationen durch die Präsenz dieser triploiden Frösche überleben, d.h. in Abwesenheit beider Elternarten. In den vergangen Jahrzenten wurden solche Populationen im Nordwestlichen Europa gründlich untersucht. Doch triploide Wasserfrösche wurden auch in Regionen in Zentraleuropa gefunden. Für diese Gebiete fehlen jedoch genaue Daten zum Paarungssystem und zur Bedeutungder verschiedenen Genotypen für die Erhaltung der jeweiligen Systeme. Daher ist das Hauptziel dieser Arbeit, a) die Paarungssysteme der Populationen in Zentraleuropa genauer zu untersuchen, b) die Paarungssysteme mit denen in reinen Hybridpopulationen in Nordeuropa zu untersuchen und c) herauszufinden, ob die triploiden Frösche in den verschiedenen Regionen mono- oder polyphyletischen Ursprungs sind.

In Kapitel eins verwendete ich Mikrosatelliten-Analysen und Kreuzungsexperimente, um fünf Populationen mit unterschiedlicher Populationsstruktur zu untersuchen. Eine Population liegt in Polen, zwei befinden in Deutschland und zwei in der Slowakei. Die Heterozygotie und genetische Differenzierung der Populationen erlaubten Rückschlüsse auf die genetische Interaktion der verschiedenen Genotypen (LLR, LR, LRR und RR). Aufgrund dieser Daten war es möglich, das Paarungssystem in jeder dieser

Populationen zu bestimmen und einen evolutionären Mechanismus für die Entstehung und den Erhalt der reinen Hybridpopulationen vorzuschlagen.

In Kapitel zwei, das in enger Zusammenarbeit mit der Dissertation von Alexandra Hoffmann entstand, wurde die Studie auf Populationen in ganz Europa ausgedehnt. Anhand von Mikrosatelliten-DNA und mitochondrialer DNA (mtDNA) suchten wir nach genetischen Unterschieden zwischen verschiedenen Paarungssystemen. Wir konnten zeigen, dass die genetische Diversität von Hybridpopulationen von der geografischen Lage (Breite und Länge) und vom Anteil der Eltern-Genotypen in der Hybridpopulation abhängen. Zudem konnten wir genetische Cluster sowohl bei den Mikrosatelliten wie auch bei der mtDNA identifizieren. Dies deutet darauf hin, dass aktuell mindestens zwei verschiedene polyploide Hybridgruppen existieren: eine im Norden und im Östlichen Zentraleuropa und eine in Osteuropa (Ostukraine).

In Kapitel drei konzentrierte ich mich auf acht tschechische und slowakische Populationen. Mittels Mikrosatelliten-Analysen, Durchfluss-Zytometrie und Kreuzungsexperimenten war es mir möglich, einen dritten und neuen Typ von Paarungssystem zu beschreiben: das "modifizierte LE-System". Populationen dieses Systems sind dadurch gekennzeichnet, dass triploide Individuen nur in der männlichen Linie vorkommen.

In Kapitel vier wandte ich einen ökologisch geprägten Ansatz an. Ich untersuchte Unterschiede in der Fitness der drei Hybridtypen (diploide LR und die beiden triploiden LLR und LRR) und der beiden Elternarten (LL und RR). Dazu setzte ich die Larven der verschiedenen Genotypen zwei verschiedenen Temperaturen aus und mass eine Reihe von life history Merkmalen. Diploide und triploide Hybriden hatten höhere Fitness bei kühleren Temperaturen. Der Befund hilft, zu erklären, wieso reine Hybridpopulationen im nördlichen Teil der Verbreitung der Art dominieren.

Kapitel fünf beschreibt ein gemeinsames Projekt mit Kolleginnen und Kollegen aus Polen. Mit Hilfe einer detaillierten Analyse von 18 Mikrosatelliten-Loci (davon einige mit Dosis-Effekten) konnten wir den Ursprung eines unerwarteten pentaploiden Nachkommen aus künstlichen Kreuzungen klären. Wir konnten nachweisen, dass der pentaploide Frosch aus der Fusion eines haploiden L-Spermiums und eines tetraploiden Eis

entstanden war, das die doppelte DNA des gesamten mütterlichen Genoms (Hybrid LR) enthielt (ein LLRR-Ei). Dieses Kapitel demonstriert eine praktische Anwendung, wie Mikrosatelliten mit Dosis-Effekten helfen, den Ploidie-Level und die Anzahl Kopien von zwei spezifischen Genomen in Hybriden, in diesem Fall *P. lessonae* und *P. ridibundus*, zu bestimmen.

Insgesamt trägt diese Studie dazu bei, die Diversität und Komplexität unterschiedlicher Wasserfrosch-Paarungssysteme mit Triploiden zu verstehen. Zweitens zeigt sie die verschiedene Herkunft von Triploiden in verschiedenen Populationen auf. Schliesslich präsentiert sie einen evolutionären Mechanismus, wie reine Hybridpopulationen entstehen und erhalten bleiben können. Solche Populationen stellen wichtige evolutionäre Einheiten dar, die die Beachtung durch Biologen verdienen, aber auch Massnahmen zu ihrem Schutz verlangen.

General introduction

The evolutionary potential of interspecific hybridization is a matter of great interest in evolutionary biology. While viewed as an important way for promoting genetic diversity and speciation in plants (Stebbins 1950, Grant 1971, Rieseberg 1997), examples of interspecific hybrid taxa that were able to successfully establish themselves are very scarce in the animal kingdom and deserve more careful investigation (Arnold 1997, Mable 2004, Mallet 2005, Mallet 2008, Schwenk et al. 2008).

Hybridization

When individuals from two species or diverse populations of a species hybridize, the produced F1 hybrid progeny usually exhibit intermediate physiological, morphological and behavioral characteristics, which often puts them at a selective disadvantage in either of the parental species' habitats. Beside this exogenous selection against hybrids, there can also be negative endogenous selection caused by the disruption of advantageous gene combinations, negative epistasis and/or chromosomal mispairing of the two parental genomes (Bronson et al. 2003, Peterson et al. 2005) resulting in unviable or infertile offspring. For these reasons, animal hybrids are usually considered as evolutionary dead ends.

Nevertheless, hybridization is common in nature and often successful in the sense that hybrid lineages occupy extensive geographical ranges, maintain large populations and have achieved considerable evolutionary longevity (see reviews in Arnold 1997 and Mallet 2005). Several models have been proposed to explain the evolutionary success and longevity of some hybrid taxa (Bullini 1994, Alves et al. 2001, Janko et al. 2003).

• According to the tension zone model (Barton and Hewitt 1985) hybrids are selected against but their presence is maintained by constant new generation in the area where the parental species overlap.

• The bounded hybrid superiority model (Moore 1977) assumes that hybrids are inferior and less fit in the parental habitat, but more fit than the parental phenotypes within a narrow ecotonal hybrid zone where they use an intermediate ecological niche.

• The mosaic model (Harrison and Rand 1989) assumes no smooth ecological transition, but patchy mosaics of different habitats in hybrid zones. The differential preferences of the hybrid and its parental species lead to a patchy distribution of genotypes in the contact zone.

According to the tension zone model the boundary between the two parental species is reinforced and the hybrid is restrained to narrow zones. In contrast, the two other models allow spatial habitat or niche partitioning which open the possibility of isolation of the involved taxa and independent evolution of the hybrids. However, for achieving such independence, ecological success is not sufficient. Hybrids also have to overcome possible difficulties in meiotic chromosome pairing at gamete production.

Sexual versus asexual reproduction

Incompatibility of the parental species' genomes often leads to drastically lowered fitness of the hybrid offspring, ranging from zygotic mortality to inviability or infertility. Some hybrid animal taxa have overcome those problems by adopting one of the three reproductive modes without recombination that are illustrated in Figure 1. In parthenogenesis unreduced eggs develop without male input; in gynogenesis an unreduced egg needs the contact with sperm to trigger the development but does not incorporate the paternal genetic material; in hybridogenesis one of the parental genomes is discarded pre-meiotically and the other clonally transmitted to gametes.

These asexual organisms are of particular interest to biologists because they allow testing hypotheses about the advantages and disadvantages of sexual versus asexual reproduction. One of the paradigms of evolutionary biology asserts that sexual reproduction is superior to asexuality because it (a) maintains polymorphism and higher genetic diversity, thus guaranteeing greater plasticity in changing environments and (b) prevents the accumulation of deleterious mutation through Muller's ratchet which, in the absence of genetic recombination, can not be purged (Muller 1964).

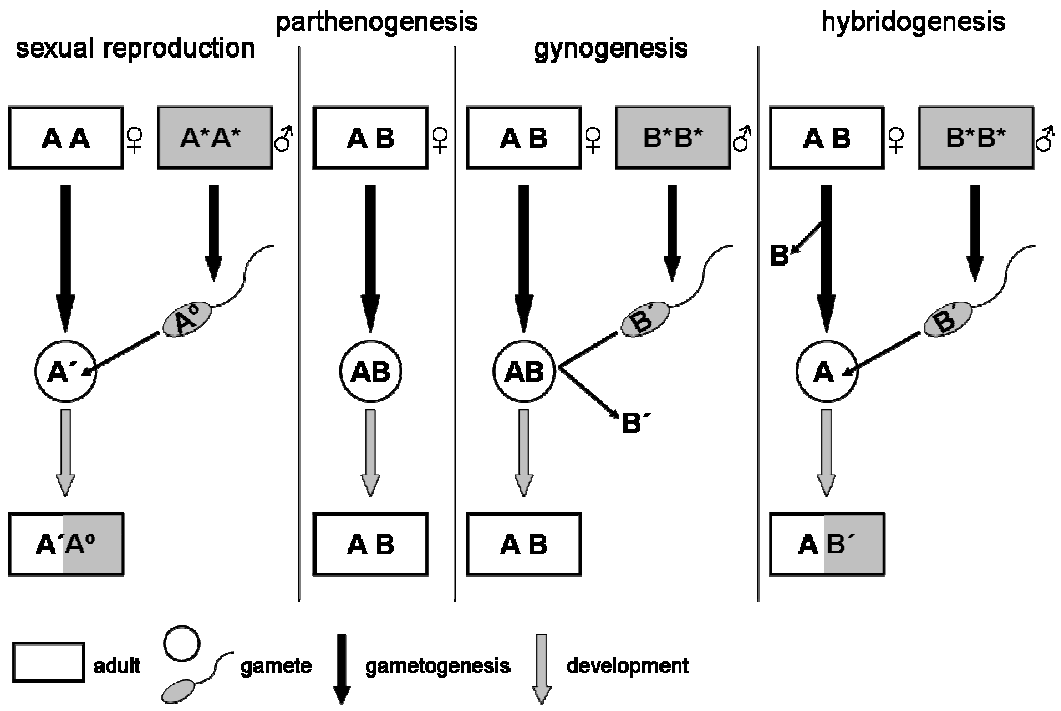

Figure 1: Reproductive modes in vertebrates (after Dawley 1989)

In asexual organisms, to be of adaptive benefit, combinations of mutations must be fixed within the same lineage, because selection acts on the entire individual rather than on alleles only (Vorburger et al. 2003). On the other hand, in sexual organisms, advantageous mutations can appear in different individuals and be combined later on through mating and recombination. These evolutionary benefits from sexual reproduction are, at least to some extent, counteracted by genetic and demographic costs. Genetic costs include the disruption of adaptive allele combinations. In terms of demography, the need to produce males represents a twofold cost compared to clonal reproduction (Neher et al. 2010).

Polyploidisation

Often, hybridization not only leads to asexual reproduction but also to allo-polyploidy, i.e. with a situation where individuals carry three or more complete sets of chromosomes from the parental species. This is due to problems during meiosis when chromosomes from different species do not pair and segregate properly and, hence, unreduced gametes are produced.

Subsequent fusion of such gametes results in an increase of the ploidy level in the offspring (Vrijenhoek 1989, Ramsey and Schemske, 1998).

This link between hybridization, asexual reproduction and polyploidisation (Choleva et al. 2012) creates genetic systems with the potential for hybrid speciation through allo-polyploidisation.

From hybridization through polyploidy to speciation

The probability of establishing an independently evolving polyploid hybrid lineage (i.e. hybrid or “reticulate” speciation) can be expected to increase with:
(1) The rate at which unreduced gametes are produced. Because of segregation problems during meiosis, interspecific hybrids are more likely to produce diploid gametes than non-hybrids.
(2) The likelihood that these unreduced gametes will fuse. This factor being determined by their frequency in the population and by the mechanisms affecting their encounter rate.
(3) The viability and fertility of the resulting allopolyploid offspring. Whether polyploid zygotes develop into viable and fertile offspring depends on genetic compatibility between the maternal and the paternal genomes.
(4) The competitive ability and the reproductive isolation of such offspring from its parental species. Polyploids (and hybrids in general) are more likely to get established and persist if they can reduce competition by inhabiting different or broader niches than their progenitors.

These four factors make some straightforward predictions about genetic, reproductive and ecological conditions that favor polyploidy and make it a promising evolutionary force in speciation. Polyploidy should be favored in species that:

- have a high rate of diploid gamete production
- have a mating system enhancing the likelihood that these gametes will fuse
- produce viable and fertile polyploid offspring in number
- show signs of recurrent polyploid origin
- occur under fluctuating or harsh conditions with reduced competition from their progenitors.

The hybridogenetic European water frog complex *Pelophylax esculentus* is showing these features and, thus, represent an excellent model

organism for studying steps in the transition from hybridization through polyploidy to speciation.

Biological model organism

The *Pelophylax esculentus* complex

The *Pelophylax esculentus* complex consist of two parental species of water frogs, *P. lessonae* (Camerano 1882) (genotype LL) also known as the pool frog and *P. ridibundus* (Pallas 1771) (genotype RR), the marsh frog, and their hybrid *P. esculentus* (Linnaeus 1758) (genotype LR), the edible frog. The hybrid reproduces trough hybridogenesis.

Hybridogenesis

This peculiar mode of reproduction was first described by Schultz (1969) in the Mexican fishes of the genus *Poeciliopsis*. Meanwhile it is also known from a few additional animal taxa, including insects (*Bacillus*, Mantovani and Scali 1992), fishes (*Squalius,* Carmona et al. 1997) and anurans (*Pelophylax*, Berger 1968) (see Dawley 1989 for a review).

Hybridogenesis is also termed “hemiclonal reproduction” because elimination of one of the parental genomes at an early stage of gametogenesis is followed by the production of gametes that clonally transmit only the other half of the parental genomes. The striking features of hybridogenesis is that during mitotic cell divisions, the chromosomes of each parental set appear to behave perfectly normally, whereas in meiosis, one of the parental chromosome sets is regularly excluded from the gametes. Little is known about the mechanisms of genome eliminations. Cimino (1972) described the formation of unipolar meiotic spindles during gametogenesis in *Poecilliopsis*, leading to the condensation of only one of the chromosome sets: the other one was spread in the cytoplasm without being gathered in a nuclear membrane. Later on, Heppich et al. (1982) proposed a model for water frogs where one genome is premeiotically excluded, followed by endoreplication of the remaining genome. At this stage, germ line cells can enter a normal cycle of meiotic divisions resulting in the production of haploid eggs or sperm containing clonal copies of only one of the parental genomes.

Again in water frogs, Ogielska (1994) observed Nucleus-Like Bodies (NLBs) in the cytoplasm of germ line cells of the hybrid, i.e. vacuoles containing DNA which could be product of the elimination of one parental genome.

A derived mechanism of genome exclusion known as “meiotic hybridogenesis” (Alves et al. 1998) is found in triploid individuals. They usually exclude the genome they have in single copy and produce haploid gametes containing the other genome after recombination (Morishima 2008, Christiansen 2009).

Breeding systems versus population composition

When genotype, ploidy and sex ratio are considered, populations of water frogs show very diverse compositions. But the sampled specimens do not always reflect the breeding interactions which occur among them. This is why I recommend to clearly distinguish between the population composition type and the breeding system type.

By population composition type I mean the taxa composition sampled in the field. A population can, for instance, be denoted as an $LE_{2n}R$-population when composed of both parental species and diploid hybrids, or as an $E_{2n}E_{3n}$-population in the case of an all hybrid population containing diploid and triploid hybrids. On the other hand, the term breeding system type should be restricted to depicting the genetic interactions that occur in these populations and allow the presence and persistence of the hybrids. At the moment, three main water frog breeding system types are described and accepted (Table 1):

• The LE-system where the hybrid excludes the L genome, produces haploid R gametes and restores hybridity by mating with sympatric *P. lessonae*.

• The RE-system, where the hybrid excludes the R genome, produces haploid L gametes, and persists by mating with sympatric *P. ridibundus*.

• The EE-system where the hybrids are present in diploid (LR) and triploid (LLR and LRR) forms. Here, diploid females are mostly producing diploid ova while the triploid individuals produce haploid gametes containing the recombined genome they have in double dose (L gametes for LLR and R gametes for LRR).

a) LE-system	**Males**	LL	LR
Females	***Gametes***	*L*	*R*
LL	*L*	LL	LR
LR	*R*	LR	RR

b) RE-system	**Males**	RR	LR
Females	***Gametes***	*R*	*L*
RR	*R*	RR	LR
(LR)	(*L*)	(LR)	(LL)

c) EE-system	**Males**	LLR		LR		LRR	
Females	***Gametes***	*L*		*R*		*R*	
LLR	*L*	LL		LR		LR	
LR	*LR*	*R*	LLR	LR	LRR	RR	LRR	RR
LRR	*R*		LR		RR		RR	

Table 1: Offspring types (within bold frame) expected from typical gamete types (in italics) and mating combinations that are possible in a) LE-systems, b) RE-systems and c) an EE-systems consisting of diploid LR and two types of triploids, LLR and LRR. Offspring types in grey fields do not occur among adults, although they are initially produced. The brackets in b) indicate that in most RE-systems female hybrids and the resulting gamete and offspring types do not occur.

The breeding system type denotes the necessary minimum of taxa in a population that allows the hybrid to persist, but it does not always reflect the exact population composition. It is possible, for instance, that an LE-system, (meaning *P. lessonae* breeding with *P. esculentus* producing R gametes) contains some *P. ridibundus* frogs. These will possibly mate with the hybrid but will not contribute to the reproduction of *P. esculentus*, because the resulting offspring (if ever they survive) will generate a new generation of *P. ridibundus*. Such a mixed population will be defined as a LER-population of the LE-system.

On the other hand, the population type gives information on the taxa which compose the population but not on the breeding system occurring

there. In the Czech Republic, for instance, we found a $LE_{2n}R$ population containing the two parental species and the hybrid but according to our crossing experiments it appears to be a population where LE- and RE-systems meet (chapter 3 of this thesis).

Approach to my research questions

Given that previous studies usually described composition of water frog populations, rather than genetic interactions, it was basically impossible to decide whether breeding systems in populations containing triploid hybrids are identical, or whether they differ between the well-studied all hybrid populations from North-Western Europe (Christiansen 2005, 2009, Christiansen and Reyer 2009, Arioli et al. 2010, Jakob et al. 2010) and populations with scanty information from Central-European regions (Berger 1988, Rybacki and Berger 2001, Mikulíček and Kotlik 2001, Plötner 2005). To allow such a comparison, this thesis focused on asking what types of recombined, respectively clonal, gametes are contributed by which genotypes and sexes in various areas.

In chapter one I sampled frogs from North-Western Europe (Germany and Poland) and Central Europe (Slovakia) and performed crossing experiments with them to determine the gamete production patterns occurring in these populations. In addition to population genetic indices, this allowed to identify three different breeding systems and to propose an evolutionary scenario for their origin and maintenance.

In chapter two, Alexandra Hoffmann and I performed an even wider population survey and, using DNA microsatellites and mitochondrial DNA analyses, we identified genetic clusters which indicate that there are at least two separate polyploid hybrid lineages existing today: one in Northern and East-Central Europe and one in Eastern Europe (Eastern Ukraine).

Chapter three presents a detailed analysis of the genetic interactions in eight populations from Slovakia and the Czech Republic, resulting in the detection of a so far undescribed breeding system containing a unique all male lineage, the “modified LE-system”.

In chapter four, I experimentally tested for ecological effects on life history traits of the three hybrid types (LLR, LR and LRR) and their two parental species (LL and RR). The outcome revealed genotype-temperature

interactions on larval performance which can shape population structure of water frogs and explain the success of all-hybrid populations at the northern border of the complex range.

Chapter five illustrates the usefulness of a very detailed DNA microsatellite analysis for detecting unusual genotypes, in this case a pentaploid hybrid (LLLRR) originating from the fusion of an L sperm with an allotetraploid LLRR ovum.

I think that this approach, from the macro (European survey and population genetics) to the micro (chromosome segregation analysis) point of view highlights the evolutionary potential of the astonishing hybridogenetic complex of the European water frogs *Pelophylax esculentus*. This system provides a unique tool to study many different open questions in evolutionary biology, such as the evolutionary significance of genetic diversity, the accumulation of deleterious mutations in clonally transmitted genomes, as well as the mechanisms of exclusion of the parental genome which could help understanding meiotic mechanisms in vertebrates in general.

Water Frog's Glossary:

- **Complex**: A group of taxa including the hybridogenetic hybrid and its two parental species. Nowadays three major complexes are recognized among the western Palearctic water frogs: the *P. esculentus* complex (*P. esculentus* being an hybrid between *P. lessonae* and *P. ridibundus*), the *P. hispanicus* complex (*P. hispanicus* hybrid between *P. bergeri* and *P. ridibundus*), and the *P. grafi* complex (*P. grafi* hybrid between *P. perezi* and *P. ridibundus*). See also synklepton.
- **Genomotype** (Lowcock 1994): "Number and specific origin of putative whole genome in an individual", equivalent to the genomic constitution of the hybrid. E.g. triploid hybrid of LLR genomotype.
- **Hemiclone** (Vrijenhoek et al. 1977): The clonally transmitted haploid genome (half of a diploid genomotype) resulting from hybridogenesis. Defined by a combination of genetic markers which allows detecting a repeated multi-locus genotype (MLG) in the population, equivalent to a MLG lineage. The presence of hemiclones in the population is giving information on the breeding system occurring in the population (presence of R hemiclone in LE-systems, of L hemiclones in RE-systems, and absence of hemiclones in EE-systems).
- **Hybrid vigor** (Moore 1977): Describes the increased fitness of hybrid offspring as a result of mixing the genetic contribution of its parents. Hybrid vigor is used when the parents are taken from different species while the term heterosis is used to describe the same situation when the parents are taken from different populations of the same species.
- **Hybridogenesis**: First described by Schultz (1969) for *Poecilliopsis*. Reproduction mode based on a mechanism of genome exclusion occurring in the germ-line tissues of taxa of hybrid origins. One of the parental genomes is exclude in the first steps of gametogenesis, probably before the first meiotic division stage (Graff and Muller 1979); the other genome is clonally transmitted. This reproductive mode requires the presence of the parental species whose genome is excluded in the population in order to perpetuate hybridity at each generation.
- **Hybridolysis** (Günther and Plötner 1988): Regeneration of one of the parental species by homotypic crosses between hybrids transmitting genomes of same specificity. In most of the populations containing *P. esculentus* such crosses give raise to unviable offspring because of the combination of hemiclones having accumulated deleterious mutations in the same loci (Muller's ratchet). Backcrossing of the hybrid with the parental species whose genome is transmitted can also lead to hybridolysis but can only happen in populations where the three species are living in sympatry or close vicinity.
- **Klepton** (Dubois and Günther 1982): Term of Greek origin meaning thief, introduced in order to allow zoological nomenclature to account for the hybrid origin and the special mode of reproduction of some hybridogenetic taxa. Klepton designates the taxon of hybrid constitution which depends at each generation on another taxon for its reproduction (e.g. *P*. kl. *esculentus*). Terminology not yet accepted by the International Code of Zoological Nomenclature (Anonymous, 1999).
- **Meiotic hybridogenesis** (Alves et al. 1998): Special case of hybridogenesis occurring in allotriploid hybrids where genomes existing in single copies are excluded and those in double dose are recombined before being transmitted to haploid gametes.
- **Monophyletic:** Characterizes a group that contains all of the descendants of a particular node in a phylogeny. Implies a unique origin.
- **Multi locus genotype** (MLG): Combination of alleles of different genetic markers (allozymes or microsatellite DNA). In the hybrids, looking at each parental genome respectively, the identification of a repeated MLG allows to identify MLG lineages hemiclonally transmitted (equivalent to hemiclones).
- **Muller's ratchet** (Muller 1964): Term first coined by Felsenstein (1974) to describe the process by which the genome of asexual populations accumulates slightly deleterious mutations in an irreversible manner.
- **Polyphyletic:** Characterizes a group containing taxa having more than one ancestor. Implies multiple origins.
- **Population type:** Used to inform about the genotypic structure or genomotypic composition of a population. Acquaint only for the frogs sampled on site, information which is not always sufficient to infer the breeding system type.

- **Primary hybridization**: Crosse between a pair of parental species leading to the generation of an array of F1 hybrids.
- **Synklepton** (Dubois and Günther 1982): Designates the three taxa composing a species complex showing an hybridogenetic mode of reproduction. E.g. *Pelophylax* synklepton *esculentus* designates the hybrid *P. esculentus* and its two parental species *P. lessonae* and *P. ridibundus*. As this terminology has not yet been accepted by the International Code of Zoological Nomenclature (Anonymous, 1999) we here recommend the use of complex instead of synklepton. See also Klepton
- **System type** (breeding system type): First used in water frogs by Uzzell and Berger (1975). Used to inform about the reproductive mode or breeding system happening in a population. LE-system, RE-system or EE-system must be defined by the genetic interactions leading to the perpetuation of the hybrid taxa taking into account which of the two genomes is passed on through the hybrid.

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Gamete production patterns, ploidy and population genetics reveal evolutionary significant units in hybrid water frogs (*Pelophylax esculentus*)

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Abstract.- The European water frog *Pelophylax esculentus* (genotype LR) is a natural hybrid between *P. lessonae* (LL) and *P. ridibundus* (RR). It reproduces through hybridogenesis, i.e. it eliminates one of the parental genome from its germline and produces gametes containing the clonally transmitted genome of the other parental species. In the north-western part of its range, *P. esculentus* is also found in triploid forms (LLR and/or LRR) that reproduce through meiotic hybridogenesis, i.e. the triploid eliminates the genome they have in single copy and produce haploid gametes containing the other genome after recombination. Triploid hybrids have also been reported for the Central-European region, but publications provide no details on the breeding system adopted by frogs there. In order to study their breeding system type, identify their gamete production pattern and to assess whether polyploidy in different area is of mono- or polyphyletic origin we sampled frogs from different populations, conducted microsatellite analyses and performed crossing experiments.

We showed that *P. esculentus* populations containing triploid specimens are different all over Europe. First they show different genomotype composition. In North-Western Europe we found all hybrid populations with diploid and both triploid types occurring in both sexes and no animals of the parental species nearby. In contrast, populations in Central Europe were composed of diploid hybrids of both sexes and triploids consisting of LLR

males only with both parental species in close vicinity. Second, populations differ in their gamete production patterns. In North-Western Europe, triploids predominantly arise from fusion of diploid LR gametes, produced by diploid LR females, with haploid recombined L or R gametes produced by LLR or LRR triploid males via meiotic hybridogenesis (E-E breeding system). Central Europe, however, exhibit a “modified L-E breeding system” where triploid LLR males perpetuate themselves by producing diploid LL sperm that fertilizes hemiclonally transmitted haploid R eggs, produced by LR females as in typical LE-systems.

Those differences in breeding systems, genetic distances and gamete production pattern by different hybrid types are strong evidence for polyphyletic origins of triploid frogs. These examples shed light on the evolutionary potential inherent to the *P. esculentus* water frog complex where rare events due to untypical gametogenetic processes can lead to the raise, the perpetuation and the dispersion of new evolutionary significant lineages.
At least in the case of the EE-systems and as quoted by Schultz (1989), we here “demonstrated that they (non Mendelian forms of hybrid origin) have evolved adaptations distinct from parental biotypes and have assumed evolutionary directions that are different and independent of them”.

Key words: Hybridization, polyploidy, hybridogenesis, *Pelophylax esculentus*, breeding system, speciation

Introduction

Fertile taxa of hybrid origin are pushing the biological species concept to its limits (Dobzhansky 1937, Mayr 1942, Mallet 2008). By allowing genetic interactions between well defined and differentiated taxa, hybrids are challenging the most acknowledged mode of speciation by divergence followed by reproductive isolation. Hence hybrids constitute biological models of high interest in evolutionary biology and represent valuable material for the ongoing debate on the definition of the nature of species (i.e. whether they are real entities or just arbitrary constructs of the human mind) and on the process of speciation (Mallet 2001, Coyne and Orr 2004, Abbott et al. 2008). They allow scrutinizing the importance of gene transfer between "good species" and the importance of polyploidisation in generating new significant evolutionary units (Arnold 1992).

Secondary contact of diverged genetic entities can lead to hybridization when it happens before effective premating barriers have developed. However, failure in segregation chromosomes from different species often leads to a tremendous fitness decrease in the hybrids' offspring, ranging from zygotic mortality to inviability or infertility. Some hybrid taxa have escaped the genetic incompatibilities and the resulting detrimental effects on fitness by abandoning normal meiosis. They have shifted from sexual to clonal genome transmission and adopted one of the following three reproductive modes:

• In parthenogenesis, offspring develop from unreduced eggs without any male input

• In gynogenesis such unreduced egg need the contact with sperm to trigger the development, but do not incorporate the paternal genetic material

• In hybridogenesis (Schultz 1969), one of the parental genomes is excluded during the first steps of meiosis, followed by the production of clonal gametes containing the other parental genome. By living in sympatry and mating with the parental species, whose genome has been excluded, hybridity is re-establish and thus a hemiclonal hybrid line perpetuated. Such a reproductive mode has been shown to exist and be quite stable in natural animal populations of insects (*Bacillus*, Mantovani and Scali 1992), fishes (*Squalius,*

Carmona et al. 1997, and *Poeciliopsis,* Schultz 1966) and anurans (*Pelophylax*, Berger 1968).

Where problems of chromosome pairing during gametogenesis lead to occasional failure or regular circumvention of chromosome segregation, and hence the production of unreduced gametes, an increase of the ploidy level of the offspring can result (Vrijenhoek 1989, Ramsey and Schemske, 1998). Thus, there is a link between hybridization, asexual reproduction and polyploidisation which creates genetic systems with the potential for hybrid speciation through allopolyploidisation.

The probability of establishing an independently evolving polyploid hybrid lineage can be expected to increase with (1) the rate and type (in terms of genomic composition) at which unreduced gametes are produced, (2) the likelihood that they will fuse, (3) the viability and fertility of the resulting allopolyploid offspring and (4) the competitive ability and the reproductive isolation of such offspring from its parental species. Chances of establishing a stable and self-perpetuating polyploid lineage are expected to be highest for even-ploidy (e.g. tetraploidization) because it allows biparental reproduction with normal meiosis. It has been shown, however, that triploid forms producing diploid gametes in one sex and haploid ones in the other sex can act as a stepping stone towards tetraploidization (triploid bridge; Ramsey and Schemske 1998, Mable 2004, Cunha et al. 2008). Moreover, as hybrids are often capable of occupying habitats beyond the limits of their diploid progenitors (Endler 1973, Moore 1977, Arnold 1997), we can expect that if such hybrids manage to produce the necessary gamete types, they can replace populations of their parental species. Thus, under certain genetic and ecological conditions hybrids can become evolutionary independent units.

The evolutionary impact of hybridization and polyploidy has been well demonstrated among plant species (Stebbins 1950, Grant 1971, Rieseberg 1997), but examples from the animal kingdom are scarce, especially when it comes to vertebrates (Arnold 1997, Mallet 2008, Schwenk et al. 2008). This is why this study focuses on the type of gametes produced in terms of ploidy and genomic composition in amphibian populations containing triploid specimens.

The *Pelophylax esculentus* complex

An excellent model system for investigating the evolutionary impact of polyploid hybrid and the associated shift from sexual to clonal genome transmissions is provided by Palearctic water frogs of the *Pelophylax esculentus* complex (formerly *Rana esculenta* until Frost 2006). The complex is composed of two parental species, *P. lessonae* (Camerano 1882), the pool frog (genotype LL), and *P. ridibundus* (Pallas 1771), the marsh frog (RR) and their inter-specific hybrid *P. esculentus* (Linnaeus 1758), the edible frog (usually LR). Hybrids of both sexes overcome problems of chromosome pairing during meiosis by excluding one of the parental genomes during the first division of gametogenesis (hybridogenesis; Schultz 1969, Graf and Müller 1979). The remaining genome is then clonally transmitted to eggs and sperm, respectively (hemiclonal transmission). In such a situation, hybridity can only be restored and hybrid lines perpetuate, if hybrids live and mate each generation with the parental species, whose genome was excluded. Thus, the hybrids are acting as sexual parasites of the parental host species.

The most widespread gamete production type of hybrids is the exclusion of the L and transmission of the clonal R genome. Where this occurs, *P. esculentus* lives in sympatry with at least *P. lessonae*, thus constituting so-called LE-systems. The mirror system, named RE-system, exists in the eastern part of the distribution range of *P. esculentus*. In this system, the R genome is excluded, which forces the hybrid to live and mate with *P. ridibundus*. What generates these two breeding systems remains a puzzle, because the exact mechanisms of genome exclusion are still not known; nor are the factors that determine which parental genome is inducing, respectively resisting, exclusions under what conditions.

In the northern parts of the species' range, especially around the Baltic Sea, a third breeding system type exists: the EE-system (Plötner 2005, Christiansen 2009, Arioli et al. 2010, Jakob et al. 2010). Here, populations consist of hybrids only, with no parental species occurring in the surrounding area. Those all-hybrid populations are composed of diploid hybrids (genome LR) and triploids of both LLR and LRR genome compositions. In this system, diploid females usually produce diploid LR gametes, whereas triploids

produce haploid gametes containing the recombined genome of the type they have in double dose (i.e. L in LLR frogs and R in LRR) through a mechanism called “meiotic hybridogenesis” (Alves et al. 1998, Morishima et al. 2008). The production of these three gamete types allows the generation and persistence of the all-hybrid populations; and differences in gamete production, rather than variation in ecological selection regimes, seem to explain why the proportions of LR, LLR and LRR frogs differ among ponds (gamete pattern hypothesis versus selection hypothesis; Christiansen et al. 2010, Embrecht and Reyer 2012).

These findings are based on intensive studies of all-hybrid populations in Denmark and southern Sweden (Christiansen and Reyer 2009, Arioli et al. 2010, Jakob et al. 2010). However, triploid hybrids have also been reported for several populations south of the Baltic Sea and in Central Europe, where they occur either with only diploid hybrids or with diploids and one or both parental species together (Berger 1988a, Tunner 1992, Mikulíček and Kotlik 2001, Plötner 2005). To investigate how these breeding systems function and whether populations containing triploid hybrids are of mono- or polyphyletic origin, we sampled five European populations from four different river basins and performed two different analyses. First, we conducted crossing experiments to analyse the types of gametes produced by the different hybrid genomotypes, i.e. the genomic constitution in terms of the number and origin of the constitutive genomes (Lowcock 1994). Second, we used microsatellite analysis to calculate population genetics parameters, such as expected heterozygosity (H_e, Nei 1978) and fixation index, (F_{ST}, Weir and Cockerham 1984). Together, the two approaches allowed us to infer the breeding systems and their similarities, respectively difference, between the different populations. Based on our results, we then discuss possible origins of the systems and the evolutionary potential they carry.

Material and methods

Populations

We sampled frogs in five populations from three European countries (Figure 1). In Poland, frogs were caught from two ponds located near Wysoka Kamieńska (53°49'18"N, 14°50'38"E, in this study referred to as Wysoka).In Germany, they originated from one pond situated 2 km south of the village of Herzberg am Harz (51°37'37"N, 10°21'15"E, Herzberg) , and from the village pond of Schönermark, near Kyritz (52°54'08"N, 12°19 '16"E, Kyritz). In Slovakia we sampled from two ponds close to the village of Šajdíkove Humence (48°38'34"N, 17°16'54"E, Šajdíkove) and from two ponds located in the village of Šaštín-Stráže (48°37'55"N, 17°08'40" E, Šaštín). Maximum distances between the five populations were 580 km in north-south and 470 km in east –west direction.

Frogs were collected by hand at night using a flashlight. They were determined for sexes and taxon on the spot according to phenotypic characteristics (Berger 1988b, Plötner 2005). In order to distinguish diploid from triploid hybrids, we took blood smears and measured erythrocyte lengths and widths under the microscope; triploid erythrocytes are significantly larger than diploid ones (Berger 1988a, Vinogradov 1990). All frogs were toe clipped for subsequent microsatellite DNA analyses in order to confirm the taxon identification and analyse genotype composition in the total sample. Thereafter, frogs were released back into the pond of origin, except for those selected for crossing experiments. These were individually marked with transponders (ID-162, AEG), separated by sex and assumed genotype and transported to the University of Zurich in cloth bags filled with rubber sponges. While sampling continued, and during the transport, the bags were showered daily with fresh water. All frogs survived the journey.

Microsatellite analysis

Precise genotype identification of the frogs sampled on site, of the frogs used as parents, as of the offspring resulting from the crosses, was achieved through microsatellite analysis. We used a piece of the tailfin (tadpoles) and a fingertip (adults and metamorphs), respectively, as source material. DNA

extraction and purification were performed using a Biosprint 96 DNA Blood Kit (Qiagen) in combination with the Biosprint 96 workstation following the supplier's protocol. The purified DNA was subjected to PCR runs with four primer mixes involving a total of 18 microsatellites primer pairs:

• Primer Mix 1A: CA1b6, Ga1a19 redesigned (Arioli et al. 2010), RlCA1b5, RlCA5 (Garner et al. 2000), Rrid064A (Christiansen and Reyer 2009)

• Primer Mix 1B: Re2CAGA3 (Arioli et al. 2010), Res16, Res20 (Zeisset et al. 2000), RlCA2a34 (Christiansen and Reyer 2009)

• Primer Mix 2A: ReGA1a23, Rrid169A, Rrid059A redesigned (Christiansen and Reyer 2009), Res22 (Zeisset et al. 2000), Rrid013A (Hotz et al. 2001)

• Primer Mix 2B: Re1Caga10 (Arioli et al. 2010), RlCA18 (Garner et al. 2000), RlCA1a27, Rrid135A (Christiansen and Reyer 2009).

Details on PCR protocols are given by Christiansen (2009) and Christiansen and Reyer (2009, 2011). PCR products were run for fragment length analysis on an ABI 3730 Avant capillary sequencer with internal size standard (GeneScan-500 LIZ), and the alleles were scored with the Genemapper software v3.7 (Applied Biosystems).

Loci Res20, RlCA2a34, ReGa1a23, RlCA1a27 and RlCA18 and were species-specific for *P. lessonae* while loci Rrid064A, Re2CAGA3, Res22, Re1CAGA10 and Rrid135A were species-specific for *P. ridibundus*. The other height microsatellite loci amplified in both L and R genomes. For these loci species-specificities of the alleles were known from previous studies (Christiansen 2005, Christiansen 2009, Arioli et al. 2010, Pruvost unpublished data). Four microsatellite loci (CA1b6, RlCA1b5, Ga1a19redesigned and Res16) showed a dosage effect allowing us to determine the ploidy of hybrids by comparing the height of the peaks (Christiansen 2005). The sum and congruence of the 18 microsatellites markers allowed the identification of the consensus genotype of each specimen.

Population genetics analyses

Because of the hybridogenetic mode of genome transmission which inhibits recombination between the *P. lessonae* (L) and *P. ridibundus* (R) genomes, all analyses were performed for each genome separately. Prior to analyses we tested the microsatellite dataset for the presence of null alleles in both

genomes using the software Micro-Checker version 2.2.3 (Van Oosterhout et al. 2004). Because the procedure implemented in Micro-Checker requires diploid data, we could apply this method only to the specimens of the two parental species and to triploid hybrids for the genome present in double copy. For haploid parental genomes, i.e. single-copy genomes of triploids and both genomes in diploids, the search for null alleles was done by simple examination of the data. The absence of allele was taken as an indication for the presence of a null allele. Null alleles were detected in two loci that amplify for both genomes, namely RlCA5 and Res16. In addition, loci RlCA2a34, ReGA1a23, Rrid169A showed the presence of null alleles in the R genome, while locus Re1CAGA10 betrayed a null allele in the L genome. After excluding these loci from further analyses, we could use the following ten loci for our calculations: CA1b6, RlCA1b5, Ga1a19redesigned, Rrid013A and Rrid059redesigned for both genomes, together with Res20, RlCA2a34, ReGA1a23, RlCA1a27 and RlCA18 for the L genome only, and with Rrid064A, Re2CAGA3, Res22, Re1CAGA10 and Rrid135A for the R genome only.

We investigated population structure by calculating the gene diversity corrected for sample size ($H_{e,}$ expected heterozygosity according to Nei 1978) and the fixation index (F_{ST}, according to Weir and Cockerham 1984) using the software SPAGeDi version 1.3 (Hardy and Vekemans 2002) which allows the combination of multiple ploidy levels in the same analysis. Again, because of the independence of the two parental genomes, expected heterozygosity was calculated separately for the L genome (H_{eL}) and for the R genomes (H_{eR}) for each frog genomotype in each of the studied populations. To compare gene diversity values between pairs of gene pools of different frog types, we applied two-tailed paired t-tests to the values for each locus. For comparisons between more than two types of frogs within a population we used analyses of variance with H_e as dependant variable and loci as fixed effect.

In order to estimate the genetic distances between each genetic pool of different frog types in each population, we calculated pairwise F_{ST} values separately for the L genomes of the LL, LR, LLR and LRR frogs and for the R genomes of the LR, LLR, LRR and RR frogs, respectively. P values for these F_{ST} were obtained by running permutation test with 10 000 iterations.

Concerning the interpretation of these values we followed the qualitative guideline proposed by Wright (1978): $0 \leq F_{ST} < 0.05$ indicate little genetic differentiation, $0.05 \leq F_{ST} < 0.15$ moderate, $0.15 \leq F_{ST} < 0.25$ great, and $0.25 \leq F_{ST}$ very great genetic differentiation.

In order to test for the presence of an isolation-by-distance pattern, we run two Mantel tests, for the L and R genomes respectively. Those tests were performed to examine potential correlations between the matrices of the recalculated pairwise $F_{ST}/(1-F_{ST})$ values and the natural logarithm of the geographical distances in km (Rousset 1997). All statistical tests were run using the program R (version 2.15.1, R Development Core Team 2012).

Crossing design

In order to determine the type of gamete produced by a given hybrid and to avoid the masking effect of potential genetic incompatibilities between hybrid genomes, we crossed each frog with at least one specimen of each parental species (*P. lessonae* and *P. ridibundus*) and with one other hybrid.

We originally had planned to cross three hybrids of each genomotype from the five populations but due to insufficient egg numbers in some females and/or failed fertilization through sperm of some males we could not systematically do this (see Table 1). For the same lack of gametes, we also did not perform crosses between parental males and females; but parental offspring resulting from such combinations are not relevant for our questions anyway.

Artificial crossing procedure

Crosses were performed following the artificial fertilization procedure by Berger et al. (1994) with minor modifications. Ovulation stimulation was triggered by the injection of a solution of LHRH fish hormone (Bachem H-7525) at 2 mg in 100 ml Holtfreter's solution. We injected 100µl per 10 g of body mass. After about 24 hours, when females were ready for laying eggs, males were euthanized in a buffered (pH 7) MS-222 solution (Sigma A-5040) at 2mg/l and their testes were removed, sliced and crushed in a Petri dish with aged tap water. Eggs were gently stripped into this sperm suspension, where they remained for about 2-3 minutes. After this period, the suspension was

rinsed into a new Petri dish where eggs of another female were added. This protocol allows the use of the same sperm solution to fertilize eggs from different females and to fertilize eggs of the same female with sperm from different males. Eggs were covered with aged tap water and checked for fertilization success, identified by a rotation that moves the black animal hemisphere to the top within the next 30-60 min. The next day, all eggs were transferred to 6 l containers with 1-2 cm of water. After two days unfertilized eggs, egg jelly and/or aborted embryos were carefully removed every two days to avoid bacterial and fungal development. After about 15 days embryos started to reach free swimming stage (stage 25, Gosner 1960) and were euthanized using the MS-222 buffered solution cited above. The offspring of a few crosses were used for other experiments (Pruvost et al. 2013) but their genotypic data could also be use for our purpose. All studied offspring reached at least stage 25.

Gamete production determination

Originally, we had planned to genotype a minimum of 35 offspring for each cross. However, due to limited egg availability, low fertilization success and/or unviable offspring, probably resulting from genetic incompatibilities, this goal was not always reached for some crosses, while for others more than 35 offspring could be genotyped (see result Table 5). After identifying the offspring genotypes, and knowing the genotypes of their mothers and fathers, we could determine the types and relative numbers of gametes produced by each of the two parents. Since each parent frog was used in more than one cross, we summed up the results obtained from all crosses involving this frog. Potential problems caused by parental infertility or genetic incompatibilities which may mask the actual gamete production would have been revealed by a differential gamete production patterns among crosses involving the same frog. This, however, was not detected.

Results

Population composition

Microsatellite analysis allowed us to determine the genomotypes of 487 adult frogs sampled in the five populations. Population compositions in terms of taxa and ploidy are shown in Table 2. In two populations (Herzberg, Šaštin) – from now on called “mixed populations” – diploid hybrid males and females occurred in sympatry with both parental species, whereas the other three populations only hybrids were found (“all-hybrid populations”), with the exception of one LL individual in Kyritz. In Šaštin, individuals of the two parental species existed in both sexes, but in Herzberg only males were captured.

The three all-hybrid populations also differed in their composition. In Kyritz and Wysoka, we caught all three possible genomotypes (LR, LLR, and LRR) in both sexes, but in Šajdíkove LRR was absent, LLR consisted exclusively of males and LR almost only of females (with the exception of two diploid males).

Populations genetic structure

Gene diversity

The mean gene diversity for the ten loci considered is shown in Table 2 for each genome separately and detailed by loci in Appendix A1. In the two mixed populations, L genome diversity (H_{eL}) did not differ between LR hybrids and parental LL (Šaštin: mean difference=0.007 ±0.032, $t_{(9)}$=0.215, p=0.834; Herzberg: m.d.=0.073 ±0.070, $t_{(9)}$=1.045, p=0.323),), nor did in Herzberg R genome diversity (H_{eR}) differ between LR and parental RR (m.d.=0.015 ±0.043, $t_{(9)}$=0.347, p=0.736); but in Šaštin it did (m.d.=0.240 ±0.050, $t_{(9)}$=4.799, p=0.001).

With respect to the all-hybrid populations, analyses of variance did not detect any differences in both H_{eL} and H_{eR} between diploid (LR) and triploid (LLR, LRR) hybrids in Wysoka and Kyritz where all three genomotypes occur (Tables 2 and 3). In contrast, in Šajdíkove, with (mostly) LR females and only LLR males, H_{eL} values differ greatly between diploids and triploids (m.d.=0.251 ±0.080, $t_{(9)}$=3.130, p=0.012), while H_{eR} values do not (m.d.=0.029

±0.016, $t_{(9)}$=-1.862, p=0.095). In this population the allelic composition of all expressed loci of the double L genome of the triploid males is exactly the same among all specimens. Microsatellite dosage effect also revealed the presence of one tetraploid male (LLRR) possessing the same double L genome as the triploids in addition to a double R genome completely homozygote for the studied loci.

Population differentiation

The overall genetic differentiations (represented by global F_{ST} values) shows substantial and highly significant differentiation among populations for both genomes, assigning 43.59% of the variation in the L genome (global F_{ST}=0.436, p<0.001) and 25.42% in the R genome (global F_{ST}=0.254, p<0.001) to inter-population differences. Part of this variation may be due to an isolation-by-distance pattern, because we found a tendency for correlations between pairwise $F_{ST}/1\text{-}F_{ST}$ values and geographic distance for both genomes (L genome: r=0.429, p=0.086; R genome: r=0.5454, p=0.063).

The pairwise F_{ST} values between each frog genomotype in each population are given in Table 4. In the two mixed populations, there is little differentiation between LR and LL in the L genome (Šaštin: F_{ST}=0.028; Herzberg: F_{ST}=0.024) and little to moderate differentiation between LR and RR in the R genome (Herzberg: F_{ST}=0.033; Šaštin: F_{ST}=0.138). Among the all-hybrid populations, differentiation is low for both genomes within Wysoka and Kyritz, where all three hybrid types occur (all $F_{ST} \leq 0.041$) In Šajdíkove, with only two hybrid types differentiation between LLR males and mostly LR females is also low for the R genomes (F_{ST}=0.008), but very high for the L genomes (F_{ST}=0.517).

Gamete production

We performed a total of 198 crosses involving 64 *P. esculentus* (35 LR, 21 LLR, and 8 LRR), 18 *P. lessonae* and 15 *P. ridibundus*. We genotyped the 97 adults crossed and 4´675 tadpoles resulting from these crosses. The results of the gametes produced are presented in Table 5.

In two populations we encountered problems which resulted in low offspring numbers or even no offspring at all (see column N off. in Table 5).

These problems concerned five LLR males and two LR females from Šajdíkove, and the only LRR male and two LR males from Wysoka. In the two females, the reason was lack of mature eggs. Among the eight males, three of them (WFB015-09, WFB015-10 and WFB016-42) appeared to be sexually immature, their phenotype and size being closer to juveniles than to adult frogs. Success of the other five males was impaired by low egg numbers of the females they were crossed with and/or low numbers of the resulting tadpoles that reached stage 25 (e.g. LLR WFB021-016 from Šajdíkove, and LR WFB02-093 and WFB02-094 from Wysoka). Overall, however, we managed to analyse the proportions of gamete types produced by every hybrid type in each population, except for LLR males from Wysoka.

In the mixed populations of Herzberg and Šaštin, hybrid LR frogs of both sexes always produced haploid gametes with a clonally transmitted R genome. Among the all-hybrid populations, the pattern was more diverse.
In Kyritz, as well as in Wysoka, diploid males also exclusively produced haploid gametes with a clonally transmitted R genome, but all diploid females produced diploid LR gametes, with the exception of one female from Kyritz (WFB014-20) which produced equal numbers of R and LR eggs. Among the triploids, the prevailing pattern was the production of haploid gametes with a recombined genome of the type that is present in two copies, i.e. L in LLR and R in LRR. Without any exception this was true for all LRR of both sexes and all LLR males, whereas in LLR females it applied to only 89% of the eggs. The remaining 11% contained diploid clonally transmitted LL genomes.

In Šajdíkove triploid males always produced diploid gametes, which clonally transmit two L genomes. The microsatellite genotyping revealed that the LL multilocus genotype of all these frogs is exactly the same in all adults males caught on site and in all the offspring produced by our crosses. The diploid males and females from this population produced only clonal haploid R gametes. The general pattern of gamete production is given in Table 6.

Discussion

The gamete production patterns found in this study confirm the expected mixture of clonally and recombining genomes travelling between different frog genomotypes. In combination with H_e and pairwise F_{ST} values, which allow estimating levels of genetic differentiation between gene pools of all frog genomotypes, we can describe the genetic interactions happening in the different populations and link them to known breeding system types occurring in water frogs. In the following, we propose an evolutionary scenario for the appearance and maintenance of these systems.

Gamete production pattern

Diploid hybrids always transmitted clonal genomes, either haploid R or diploid LR. The production of haploid gametes with clonal R genomes is in accordance with the hemiclonal transmission mode expected in LE-systems (Figure 2), where the previously excluded L genome is regained by mating with *P. lessonae*, and thus hybridity restored. In contrast, the production of diploid gametes carrying clonal copies of the entire LR maternal genome is a feature expected of diploid females from all-hybrid populations of the EE-system (Figure 4) (Christiansen 2009). Here, the L and R genomes that are necessary for maintaining all three hybrid types in the population (LR, LLR, LRR) are provided by triploids that produce recombined haploid gametes of the type that is present in two copies (Christiansen and Reyer 2009, Morishima et al. 2008). With the slight modification in two Kyritz LLR females which produced a few diploid gametes containing their two L genomes, this was the pattern found in triploid frogs from Kyritz and Wysoka.

While these results confirm those from previous studies, the gamete production pattern in LLR males from Šajdíkove, with clonally produced sperm containing their double L genomes, suggests a previously not described “modified LE-system” (Figure 3) (see also chapter 3 of this thesis). Below, we discuss the three breeding system in more detail.

Breeding systems

LE-systems (Figure 2)

In our study, the LE-system, with clonal R gamete production by diploid hybrids and repeated provisioning of recombined L gametes by individuals of the parental species *P. lessonae*, is represented by the populations in Herzberg and Šaštin. In such systems, the hybrids are sexual parasites of the *P. lessonae* parental species and act as a sink for the L genome which is discarded prior to gametogenesis (Schmidt 1993, Joly 2001, Lehtonen et al. 2013).

In Šaštin, this genome transmission mode is reflected by the results from our population genetic analyses (Tables 2 and 4). In terms of the R genome, gene diversity (H_{eR}) in RR frogs (with recombination) is higher than in the LR frogs (with no recombination), and there is moderate genetic differentiation between LR and RR frogs (F_{ST} = 0.138). In contrast, gene diversity in the L genome (H_{eL}) is equally high for LR and LL frogs and genetic differentiation between their genomes is low (F_{ST} = 0.028).

In Herzberg the situation appears a bit different regarding the role of the sympatric *P. ridibundus* frogs. The relatively low genetic differentiation of the R genome between LR and RR frogs and the quite similar values of gene diversity are hints of close interactions between the two gene pools. This difference between the two populations is probably due to a higher rate of primary hybridization in Herzberg.

Modified LE-system (Figure 3)

In Šajdíkove the gamete production pattern of the diploid hybrids is the same as the one occurring in LE-systems, but this population also contains triploid hybrid LLR males, which always produce diploid LL gametes containing identical copies of the two same genomes. This mode of transmission is clearly reflected by the population genetic indices:

• First, gene diversity in the L genomes is significantly lower in LLR frogs (H_{eL}=0.201) which receive a clonal LL genome than in LR frogs (H_{eL}=0.452), where the value is similar to those of LL and LR frogs from LE-systems (Table 2). This indicates that diploid hybrids in Šajdíkove received recombined L

genomes, but the origin of these genomes is, so far, unknown, because no *P. lessonae* were found in the sampled pond. They may occur in ponds nearby.

• Second, the F_{ST} value estimating the differentiation of the L genome between LLR and LR frogs within Šajdíkove is very high.

The triploid males that transmit their double L genome and mate with diploid LR females producing R eggs sire offspring of their own genomotype. Hence, they form a unique paternal hemiclonal lineage with a frozen L genome. Since these LLR frogs exclude the R genome at gametogenesis, they are acting as a sink for the R genome, which is transmitted by LR frogs that, in turn, are acting as a genetic sink for the L genome (Figure 3). Given that the L genome of the diploids must come from another source (see above), the triploid males in the population are not essential to the perpetuation of the diploids in the breeding system. They just seem to have found a way to persist by parasitizing the R genomes of the sympatric LR hybrids. In contrast to EE-systems, which could not exist without triploids (see below), LLR males in Šajdíkove can be seen as a mere add-on to the L-E system. We, therefore, decided to name such breeding system "modified LE-system". This breeding system type is not restricted to this north-eastern Slovak population. Some triploid LLR males carrying the same two genomes (with only a 2 bp difference in one allele out of the 18 microsatellite loci) have also been found in populations from the North-Western Czech Republic, 130 km north, in the locality of Borovec (see chapter 3 of this thesis).

EE-systems (Figure 4)

The gamete production pattern of frogs from Kyritz and Wysoka corresponds to the EE-system that was intensively studied and described for Denmark and southern Sweden by Christiansen and Reyer (2009), Arioli et al. (2010) and Jakob et al. (2010). In such systems, the three different hybrid genomotypes manage to produce all the gamete types needed for their coexistence without requiring the presence of any of the two parental species. This genetic functioning is perfectly reflected in the two population genetics parameters we used. In both populations the gene diversity values for both genomes are in the same range for the three frog genomotypes (Table 3). Pairwise F_{ST} values

within populations also demonstrate very little genetic differentiation between the three genomotypes. In such breeding systems all frog genomotypes depend on each other to be produced (Figure 4):

• LR frogs arise from the combination of L gametes, exclusively produced by LLR frogs, with R gametes produced by LRR specimens, LR males and (in smaller proportion) LR females.

• LLR frogs mainly arise from fertilization of LR eggs produced by LR females with L sperm from males of their own genomotype, or (in smaller proportions) by fusion of R sperm coming from LRR and LR males with LL eggs from females of their own genomotype.

• LRR frogs only arise from the combination of LR eggs from LR females and R sperm produced by LR and LRR males.

Thus, LR and LLR frog types are absolutely necessary to the system in their role as producers of LR and L gametes, respectively, whereas LRR frogs are crucial as producers of R gametes, especially R eggs which only rarely are produced by LR females. Under these conditions, the EE-system would collapse if one of the actors would be removed. As predicted by the model of Som and Reyer (2006), such EE-system can persist under random mating: The recombination happening in the triploids provides genetic diversity equivalent to the one found in sexual populations, giving such systems an evolutionary potential comparable to that of sexually reproducing populations.

Origins and evolutionary potential of systems involving triploid hybrids

The difference of gamete production patterns, leading to the existence of triploid specimens in Wysoka and Kyritz on the one hand and in Šajdíkove on the other strongly suggest a polyphyletic origin of triploid frogs in EE- and modified LE-systems. Both systems may have developed from the most wide-spread typical LE-system (Figure 2), because all three systems are identical in that LR males produce clonal haploid R gametes; but then differences arose from the mechanisms that lead to the production triploid individuals: fusion of LR eggs from LR females with haploid sperm in the EE-system as opposed to fusion of haploid eggs with LL sperm from LLR males in the modified LE-system. The perfect identity of the two L genomes present in triploid LLR

males from the modified LE-system suggests that this lineage probably arose from a single event of L genome doubling that generated an array of clones, or even one single triploid specimen. Unraveling the origin of such frogs would demand a much broader population genetics investigations. However, whatever their origin, the 3n males in this system do not participate in the generation of the two other frog types (LL and LR). They only exploit R genomes from the pool of eggs produced by LR females and use their own double L genome to procreate themselves. They act as a sink for the R genome which already parasitizes the parental species sexual L genome. Thus, the triploids could disappear without harming the persistence of the other frog types, thus leaving an intact LE-system behind.

Concerning the EE-systems, the initial step away from the typical LE-system must have been a suppression of L genome exclusion in LR females, resulting in the clonal transmission of LR, rather than R genomes. Once produced, these 2n eggs automatically lead to both types of triploids: mating with *P. lessonae* males produces LLR offspring and mating with diploid *P. esculentus* hybrids produces LRR offspring. Due to the so-called meiotic hybridogenesis mechanism (Alves et al. 1998, Cuhna et al. 2008), LLR frogs are then able to produce recombined haploid L gametes and thus replace *P. lessonae* frogs, while LRR frogs can act as haploid R gamete donors and – in case of females – adopt the role previously fulfilled by LR females which now produce diploid LR eggs.

With the *P. lessonae* parental species having lost its essential position in maintaining the system, the hybrids become independent from the parental species and can disperse into environments where *P. lessonae* is absent. This fact, plus differential ecological tolerance permits the hybrid to establish all-hybrid populations (EE-system). In fact, the better performance of hybrids compared to the parental species under cold conditions, offers a possible explanation why the EE-system is wide-spread in colder region like the north of Europe (Negovetic et al. 2001, Pruvost et al. 2013),

This scenario highlights the high evolutionary potential of this seemingly flawed water frog system. What at first glance appears to be a failure of the typical gamete production pattern can, in situations where its meets favorable ecological condition, lead to completely new and evolutionary

significant population types and breeding systems capable of colonizing new geographical ranges. Natural events and/or introduction may have led to some more population types and breeding systems with unusual combinations of different gametes donor types. Therefore, further detailed studies of the European water frog group seem justified and promising.

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Tables

Table 1: Population composition, in term of number of frogs caught and number of frogs crossed per genomotypes, for the five studied populations. “-“ indicates for the absence of frogs of such type, “x” indicates for frog types which were present in the population but not crossed. Some of the parental species specimens used came from other populations and are not listed here.

Population		Genomotype									
		LLR		LR		LRR		LL		RR	
		♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
Herzberg	Caught	-	-	6	19	-	-	-	10	-	25
	Crossed	-	-	3	3	-	-	-	X	-	X
Šaštin	Caught	-	-	43	27	-	-	1	27	13	15
	Crossed	-	-	5	5	-	-	x	4	2	3
Šajdíkove	Caught	-	91	30	2	-	-	-	-	-	-
	Crossed	-	14	5	1	-	-	-	-	-	-
Kyritz	Caught	7	19	34	25	24	12	-	1	-	-
	Crossed	2	3	3	3	3	3	-	x	-	-
Wysoka	Caught	3	14	17	10	7	6	-	-	-	-
	Crossed	X	2	2	5	1	1	-	-	-	-

Table 2: Mean gene diversity corrected for sample size, Nei 1978 (H_e) for *P. lessonae* genomes (H_{eL}) and *P. ridibundus* genomes (H_{eR}) in the different frog types (LL, LLR, LR, LRR and RR). Sample size is given in brackets.

Pop.	H_{eL}				H_{eR}			
	LL	LLR	LR	LRR	LLR	LR	LRR	RR
Herzberg	**0.441** (10)	**-**	**0.368** (25)	**-**	-	**0.380** (25)	**-**	**0.395** (25)
Šaštin	**0.428** (28)	**-**	**0.421** (70)	**-**	**-**	**0.384** (70)	**-**	**0.625** (28)
Šajdíkove	**-**	**0.201** (91)	**0.452** (32)	**-**	**0.432** (91)	**0.402** (32)	**-**	-
Kyritz	**-**	**0.321** (26)	**0.300** (59)	**0.284** (36)	**0.358** (26)	**0.404** (59)	**0.401** (36)	**-**
Wysoka	**-**	**0.240** (17)	**0.221** (27)	**0.212** (13)	**0.512** (17)	**0.554** (27)	**0.609** (13)	-
Mean	**0.434**	**0.254**	**0.352**	**0.248**	**0.434**	**0.425**	**0.505**	

Table 3: Results from four analyses of variance relating gene diversity values for each locus to frog genomotypes for the L and the R genome respectively in Kyritz and in Wysoka.

Source	*Sum of squares*	*df*	*F value*	*P*
L genome in Kyritz	0.0070	2	0.803	0.463
R genome in Kyritz	0.0134	2	1.237	0.314
L genome in Wysoka	0.0042	2	0.238	0.790
R genome in Wysoka	0.0102	2	3.368	0.057

Table 4: Pairwise F_{ST} values using Weir and Cockerham calculation (1984). Values for the R genomes are above the diagonal and values for the L genomes under it. $0 \leq F_{ST} < 0.05$ indicate little genetic differentiation, $0.05 \leq F_{ST} < 0.15$ moderate (light green for L and light orange for R), $0.15 \leq F_{ST} < 0.25$ great (green for L and orange for R), $0.25 \leq F_{ST}$ very great genetic differentiation (dark green for L and dark orange for R) (Wright, 1978).

L\R	HerLL	HerLR	HerRR	KyrLLR	KyrLR	KyrLRR	SajLLR	SajLR	SasLL	SasLR	SasRR	WysLLR	WysLR	WysLRR
HerLL	x	-	-	-	-	-	-	-	-	-	-	-	-	-
HerLR	0.024	x	0.033	0.347	0.358	0.363	0.331	0.374	-	0.394	0.260	0.296	0.260	0.287
HerRR	-	-	x	0.378	0.375	0.379	0.314	0.362	-	0.392	0.274	0.333	0.294	0.313
KyrLLR	0.437	0.444	-	x	0.041	0.036	0.298	0.330	-	0.322	0.241	0.247	0.235	0.251
KyrLR	0.464	0.470	-	0.016	x	0.000	0.311	0.344	-	0.335	0.250	0.249	0.233	0.237
KyrLRR	0.461	0.470	-	0.040	0.017	x	0.300	0.327	-	0.326	0.255	0.240	0.227	0.228
SajLLR	0.634	0.655	-	0.616	0.625	0.643	x	0.008	-	0.095	0.148	0.213	0.207	0.213
SajLR	0.353	0.353	-	0.275	0.311	0.311	0.517	x	-	0.062	0.155	0.225	0.209	0.218
SasLL	0.371	0.374	-	0.213	0.233	0.213	0.522	0.091	x	-	-	-	-	-
SasLR	0.352	0.351	-	0.215	0.239	0.224	0.506	0.087	0.028	x	0.138	0.276	0.262	0.271
SasRR	-	-	-	-	-	-	-	-	-	-	x	0.148	0.144	0.125
WysLLR	0.499	0.501	-	0.167	0.193	0.210	0.667	0.361	0.272	0.293	-	x	0.000	0.000
WysLR	0.520	0.525	-	0.138	0.170	0.189	0.669	0.361	0.267	0.288	-	0.003	x	0.000
WysLRR	0.464	0.494	-	0.109	0.152	0.179	0.683	0.319	0.244	0.267	-	0.000	0.000	x

Table 5: Gamete production of the crossed frogs. “Population” stands for the name of the population of origin, “Geno.” for the genotype of the parent, “Ind. Numb.” for its specimen number, “N cross” for the number of crosses involving this frog, “N off.” for the number of offspring genotyped and “Gamete type” for the genomic composition and ploidy of the gametes produced.

Population	Geno.	Sex	Ind. numb.	N cross	N off.	Gamete type		
Herzberg	LR	F	WFB021-27	3	99	100 R		
			WFB021-28	4	103	100 R		
			WFB021-29	3	108	100 R		
		M	WFB021-12	5	73	100 R		
			WFB021-21	3	40	100 R		
			WFB021-22	3	57	100 R		
Kyritz	LLR	F	WFB014-21	3	74	91.9 L		8.1 LL
			WFB014-62	11	356	86.2 L		13.8 LL
		M	WFB014-55	4	173	100 L		
			WFB014-56	6	208	100 L		
			WFB014-59	7	143	100 L		
	LR	F	WFB014-20	7	12	50 R	50 LR	
			WFB014-25	10	468		100 LR	
			WFB014-63	7	158		100 LR	
		M	WFB014-05	7	303	100 R		
			WFB014-14	4	79	100 R		
			WFB014-48	6	156	100 R		
	LRR	F	WFB014-24	9	284	100 R		
			WFB014-26	7	272	100 R		
			WFB014-67	7	264	100 R		
		M	WFB014-11	6	161	100 R		
			WFB014-49	7	274	100 R		
			WFB014-58	4	143	100 R		
Šajdíkove	LLR	M	WFB007-93	4	86			100 LL
			WFB008-14	3	93			100 LL
			WFB015-13	4	10			100 LL
			WFB015-55	5	178			100 LL
			WFB015-56	2	11			100 LL
			WFB015-57	2	25			100 LL
			WFB021-16	3	3			100 LL
			WFB021-17	3	21			100 LL
			WFB021-18	3	16			100 LL
			WFB008-16	2	0			
			WFB015-09	6	0			
			WFB015-10	4	0			
			WFB021-19	2	0			
			WFB016-42	7	0			
	LR	F	WFB021-24	5	104	100 R		
			WFB021-30	3	96	100 R		
			WFB007-91	2	30	100 R		
			WFB021-25	1	0			
			WFB021-26	1	0			
		M	WFB007-90	2	8	100 R		
Šaštin	LR	F	WFB007-33	1	8	100 R		
			WFB007-35	1	12	100 R		
			WFB007-37	4	141	100 R		
			WFB015-72	8	283	100 R		
			WFB015-73	7	161	100 R		
		M	WFB007-52	4	101	100 R		
			WFB007-54	5	79	100 R		
			WFB015-03	6	84	100 R		
			WFB015-04	4	133	100 R		
			WFB015-06	7	254	100 R		
Wysoka	LLR	M	WFB003-02	2	66	100 L		
			WFB003-04	1	29	100 L		
	LR	F	WFB002-80	4	22		100 LR	
			WFB002-81	2	45		100 LR	
		M	WFB002-88	2	64	100 R		
			WFB002-92	2	66	100 R		
			WFB002-93	2	3	100 R		
			WFB002-94	1	1	100 R		
			WFB003-06	1	0			
	LRR	F	WFB002-74	4	6	100 R		
		M	WFB002-91	1	0			

Table 6: Gamete production of the different genomotypes of hybrids and inferred breeding systems in the five studied populations. Gamete types in parentheses are produced in small proportions.

Population	Genomotype						Inferred breeding system
	LLR		LR		LRR		
	Female	Male	Female	Male	Female	Male	
Herzberg	**-**	**-**	**R**	**R**	**-**	**-**	L-E
Šaštin	**-**	**-**	**R**	**R**	**-**	**-**	L-E
Šajdíkove	**-**	**LL**	**R**	**R**	**-**	**-**	Modified L-E
Kyritz	**L** (LL)	**L**	**LR** (R)	**R**	**R**	**R**	E-E
Wysoka	**L**	**L**	**LR**	**R**	**R**	**R**	E-E

Figures

Figure 1


Wysoka
Kyritz
Herzberg
Poland
Germany
Šaštin
Sajdíkove
Slovakia

Figure 2

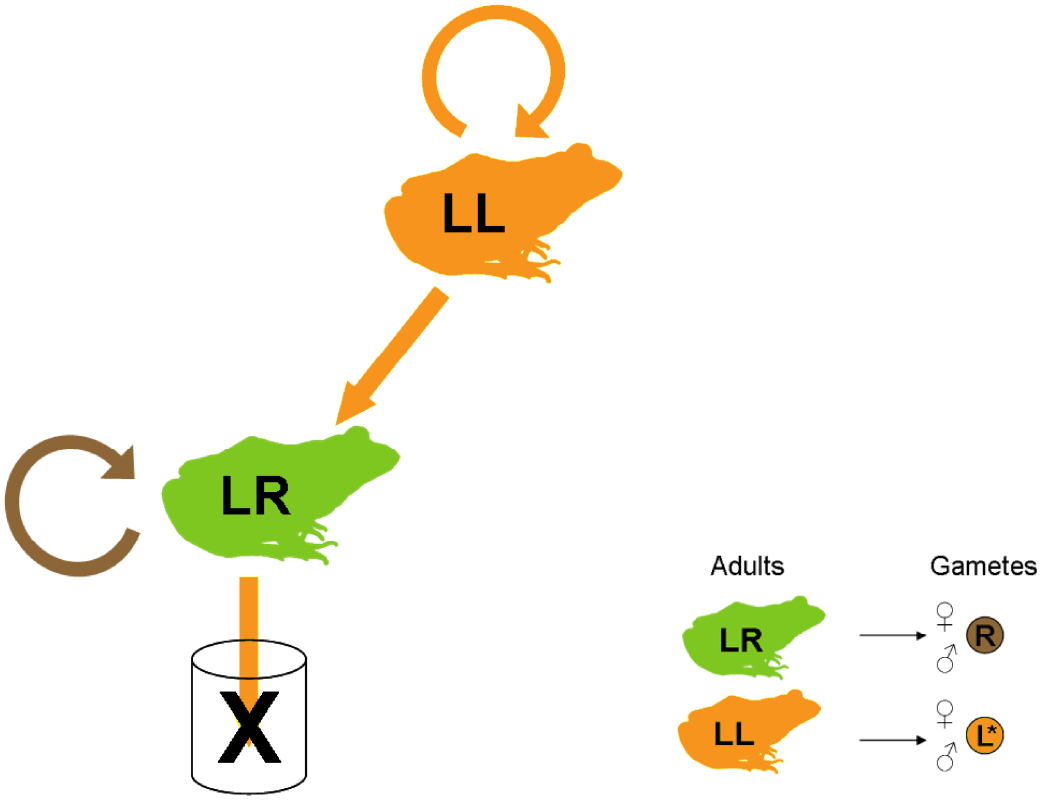


Figure 3

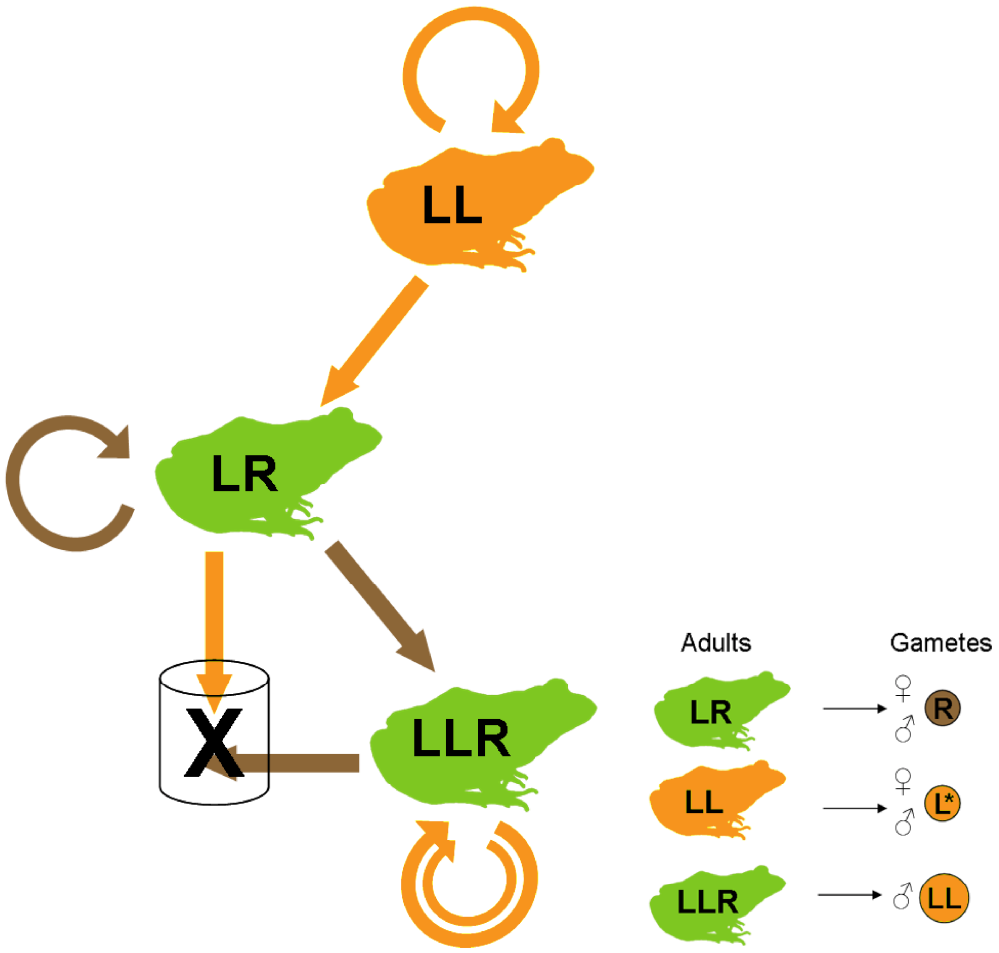
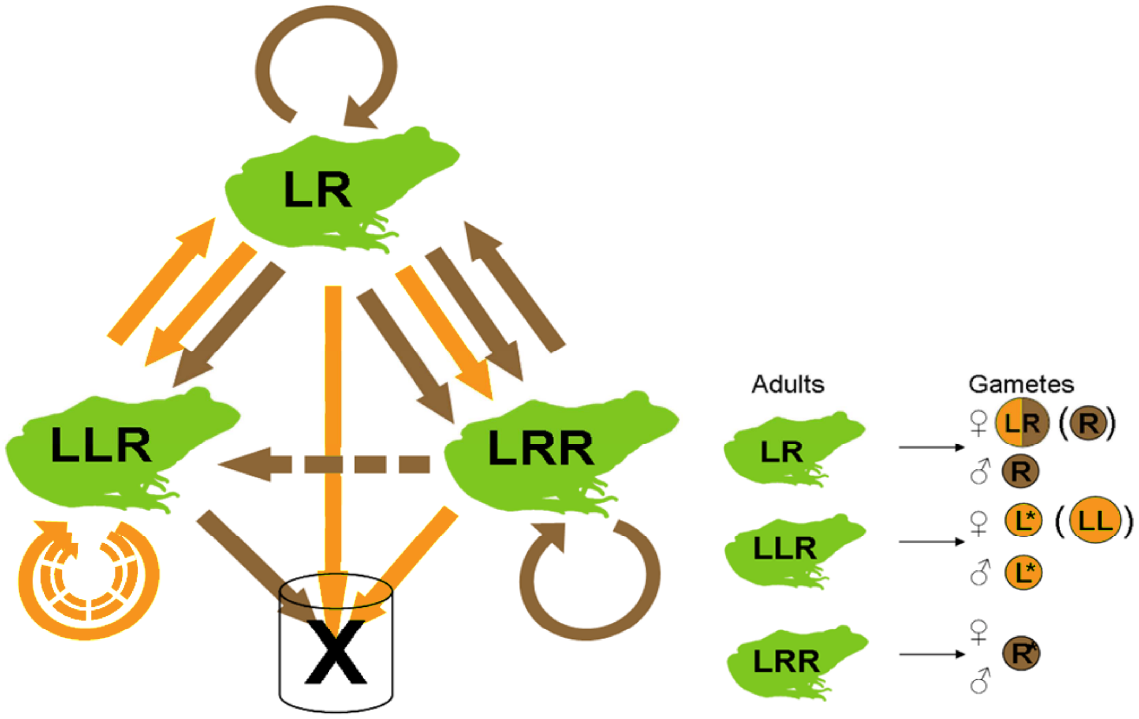


Figure 4



Appendix:

A1: Gene diversity corrected by sample size (Nei 1978) for each locus in the different frog types, for the L and the R genome respectively.

Population			All	Herzberg			Kyritz			Sajdikove		Sastin			Wysoka		
Genomotype			All	LL	LR	RR	LLR	LR	LRR	LLR	LR	LL	LR	RR	LLR	LR	LRR
N			434 L, 449 R	10	25	25	26	59	36	91	32	28	70	28	17	27	13
L genome	Locus	CA1b6	0.415	0.479	0.513	-	0.000	0.000	0.000	0.503	0.498	0.486	0.448	-	0.000	0.000	0.000
		RICA1b5	0.136	0.337	0.347	-	0.000	0.000	0.000	0.000	0.446	0.308	0.248	-	0.000	0.000	0.000
		Ga1a19red.	0.084	0.505	0.520	-	0.000	0.000	0.000	0.000	0.000	0.000	0.000	-	0.000	0.000	0.000
		Res20	0.685	0.668	0.417	-	0.652	0.521	0.348	0.000	0.665	0.203	0.472	-	0.000	0.000	0.000
		RICA2a34	0.838	0.468	0.587	-	0.741	0.831	0.833	0.503	0.665	0.785	0.755	-	0.212	0.268	0.000
		ReGa1a23	0.861	0.747	0.720	-	0.835	0.842	0.746	0.503	0.843	0.868	0.851	-	0.711	0.568	0.500
		Rrid013A	0.537	0.633	0.000	-	0.266	0.303	0.246	0.000	0.121	0.405	0.380	-	0.148	0.268	0.000
		Rrid059Ared.	0.034	0.000	0.000	-	0.000	0.000	0.000	0.000	0.000	0.000	0.000	-	0.369	0.268	0.500
		RlCA1a27	0.772	0.000	0.080	-	0.713	0.505	0.610	0.000	0.623	0.597	0.478	-	0.649	0.690	0.833
		RICA18	0.658	0.568	0.493	-	0.000	0.000	0.056	0.503	0.663	0.634	0.581	-	0.308	0.143	0.282
	Mean		0.502	0.441	0.368	-	0.321	0.300	0.284	0.201	0.452	0.428	0.421	-	0.240	0.221	0.212
	Standard Error		0.101	0.082	0.081	-	0.117	0.111	0.105	0.082	0.096	0.096	0.089	-	0.085	0.078	0.096
R genome	Locus	CA1b6	0.685	-	0.453	0.497	0.492	0.439	0.453	0.608	0.502		0.665	0.795	0.331	0.336	0.563
		RICA1b5	0.237	-	0.220	0.040	0.271	0.402	0.351	0.000	0.000	-	0.000	0.346	0.485	0.484	0.492
		Ga1a19red.	0.500	-	0.280	0.078	0.077	0.345	0.263	0.452	0.353	-	0.162	0.638	0.471	0.647	0.668
		Rrid064A	0.644	-	0.513	0.509	0.271	0.129	0.108	0.602	0.554	-	0.111	0.349	0.603	0.711	0.750
		Re2CAGA3	0.880	-	0.663	0.691	0.754	0.712	0.691	0.788	0.805	-	0.712	0.768	0.757	0.852	0.855
		Res22	0.545	-	0.280	0.393	0.077	0.230	0.309	0.510	0.444	-	0.487	0.811	0.500	0.490	0.607
		Rrid013A	0.077	-	0.280	0.458	0.000	0.000	0.000	0.000	0.000	-	0.000	0.280	0.000	0.000	0.000
		Rrid059Ared.	0.475	-	0.347	0.509	0.409	0.471	0.477	0.022	0.063	-	0.412	0.800	0.539	0.563	0.607
		Re1CAGA10	0.825	-	0.380	0.274	0.745	0.723	0.714	0.691	0.655	-	0.576	0.791	0.875	0.899	0.865
		Rrid135A	0.726	-	0.380	0.497	0.480	0.588	0.643	0.644	0.647	-	0.716	0.670	0.564	0.563	0.679
	Mean		0.559	-	0.380	0.395	0.358	0.404	0.401	0.432	0.402	-	0.384	0.625	0.512	0.554	0.609
	Standard Error		0.080	-	0.042	0.065	0.085	0.075	0.076	0.097	0.092	-	0.092	0.068	0.075	0.082	0.078

Genetic diversity and distribution patterns of diploid and polyploid water frogs (*Pelophylax esculentus*) across a large area of Europe

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Abstract.- Allopolyploid hybridization is a rare, yet sometimes successful way in animals and plants to increase diversity by creating new geno- and phenotypes that manage to extend into new habitats or to adapt to environmental changes. The hybrid water frog *Pelophylax esculentus* resulted from hybridization between two distinct water frog species (*P. lessonae* and *P. ridibundus*) which probably crossed repeatedly during interglacial periods of the Pleistocene. Today *P. esculentus* is widespread in Europe and occurs in exclusively diploid mixed populations with one of the parental species, but also in polyploid populations with or without parental species. The distribution of these polyploid populations is patchy. This study investigates variation in genetic diversity between types of *P. esculentus* populations in respect to geographic location and the presence of the two parental species. In addition, we aim to identify genetic structuring and differentiation among populations containing polyploids through combined analysis of microsatellite markers and mitochondrial DNA (mtDNA). Our results show that genetic diversity is strongly structured by latitude and longitude, increasing from north to south and – though not as strongly – from west to east. Moreover, the presence of parental genotypes positively influences genetic diversity. Analyses of microsatellite profiles identified several major genetic clusters, which widely

correspond to the patchy geographic distribution of polyploids, and yielded similar results between the two parental genomes (except for few areas where L- and R- clusters were not congruent). We discuss these results together with distribution patterns of mtDNA and relate them to possible hybridization and postglacial re-colonization scenarios.

Key words: Hybridization, polyploidy, hybridogenesis, *Pelophylax esculentus*, isolation by distance

Introduction

Natural hybridization and genetic diversity

In an ever changing environment, natural selection is the force that shapes the diversity of life forms, and genetic diversity is the raw material selection can act upon. There are many evolutionary mechanisms that create or reduce variability in genomes, most of them through an interaction of genetic and environmental processes and natural historical events. While some mechanisms and events result in genetic drift, loss of genetic diversity and subsequently in the decline of populations (Amos and Balmford 2001), others can increase genetic diversity and offer populations the potential to use new resources, expand into new habitats and evolve into new species.

Interspecific hybridization is an evolutionary mechanism that has long been considered evolutionary unimportant and maladaptive, but with new insights and a change of view on traditional species concepts, hybridization has been rehabilitated as another pathway that can increase genetic diversity and even lead to speciation (Arnold 1997, Mallet 2007). Many contemporary plants and animals show genetic evidence of past hybridization and introgression events (Arnold 1997, Dowling and Secor 1997), and some of these events resulted in stable hybrid taxa that have persisted over long periods of time. Often interspecific hybrid taxa have evolved genetic or genomic adaptations to circumvent meiotic disturbance during gametogenesis of heterozygote genomes, e.g. clonal reproduction in parthenogenetic,

gynogenetic or hybridogenetic organisms, or through the production of diploid gametes and allopolyploid offspring. Allopolyploid hybrids have long been considered to be extremely rare in animals (Mable 2004), but over the past decades more and more independent allopolyploid taxa have been found to exist in vertebrates, i.e. in several genera of fish and amphibians (Gerhardt et al. 1994, Haddad et al. 1994, Ptacek et al. 1994, Becak and Becak 1998, Alves et al. 2001, Martino and Sinsch 2002, Holloway et al. 2006, Vrijenhoek 2006, Choleva et al. 2008). Polyploidy can thus be considered another stepping stone towards speciation and although it might not be the most common way among vertebrate taxa, for some it might have been just the right way at the right evolutionary time.

Patterns of genetic diversity across Europe

In Europe, one evolutionary significant time was the series of ice ages and interglacials of the late Pleistocene (130’000 – 10’000 years ago). This period had a strong impact on the diversity and distribution of species we find today (Taberlet et al. 1998). During strong climatic oscillations, many species moved their distribution range between higher and lower latitudes and retreated to smaller refugia in the south or southeast of Europe during glacial periods, where genetic sister lineages could evolve and hybridize again during later expansion (Hewitt 1999, Hewitt 2011). Since recolonization was mostly a repetitive process, with fast northward expansion from the refugia during warm periods and subsequent contractions of range during cold periods (Hewitt 1996), successive genetic bottlenecks and loss of genetic diversity are probably the reason why we observe a negative gradient of genetic diversity from north to south in many European species (Hewitt 1999). Additional to a latitudinal genetic diversity cline, some continental species that used eastern refugia show a longitudinal genetic diversity gradient from a more diverse east towards a genetically poorer west (Schmitt 2009). These recolonization patterns have furthermore created contact or ‘suture’ zones (i.e. a geographical band of range overlap) between species that have genetically diverged during glacial periods and hybridized again during postglacial expansion (Remington 1968, Taberlet et al. 1998).

Distribution and ecology of *P. esculentus* and its parental species

For our investigation we chose a widely distributed European amphibian taxon that unites hybrid origin, allopolyploidy, geographic and genetic variety, hybridogenetic and sexual reproduction: the edible frog *Pelophylax esculentus*. *P. esculentus* is a natural hybrid of two European water frog species, the pool frog *P. lessonae*, and the marsh frog, *P. ridibundus*. The two parental species *P. lessonae* and *P. ridibundus* can be considered true continental species, with distributions extending from France as far as Russia (*P. lessonae*) and from the Rhine valley far into the Caspian Sea area (*P. ridibundus*) (Plötner 2005). Both species' distributions do not extend into Northern Europe, although a small and isolated metapopulation of *P. lessonae* exists in Sweden (Sjögren 1991), and some *P. ridibundus* populations occur at higher latitudes in the Baltic States. While *P. ridibundus* is widely distributed in the Eastern Mediterranean Sea and around the Black Sea area, *P. lessonae* meets its southern distribution boundaries in Italy, where it overlaps in a contact zone with its sister species, the Italian pool frog *P. bergeri* (Plötner 2005). Molecular evidence indicates that Italy was the main glacial refugium from where *P. lessonae* subsequently recolonized northwards, probably following a colonization route bifurcating westwards and northwards after the passage of the Alpine-Carpathian gap (Zeisset and Beebee 2001, Snell et al. 2005). *P. ridibundus* probably expanded from a refugium in the Balkan (Pagano et al. 2001). Hybridization between the two species possibly occurred repeatedly before the Pleistocene period and during Pleistocene interglacials (Uzzell 1982).

The edible frog is not a "normal" hybrid in the sense of traditional species concepts that considered interspecific hybrids evolutionary dead ends (reviewed in Dubois 2011). In fact, *P. esculentus* is one of the most common and wide-spread amphibian taxon in Europe. Its distribution range overlaps with or even extends the ones of its two parental species (Plötner 2005). In wide parts of its distribution, *P. esculentus* occurs only in sympatry with one of its parental species. In a reproductive mode called hybridogenesis (Schultz 1969), hybrids exclude one of their heterospecific chromosome sets during gametogenesis (either the "R" set inherited from *P. ridibundus*, or the "L" set

from *P. lessonae*), back-cross with the sympatric parental species (which carries the genome part the hybrid excluded) and thus regaining the excluded parental genome to produce a new generation of heterospecific hybrids. The retained part of the genome is passed on clonally, and the newly produced hybrids are hemiclones (Dawley 1989) that are usually unable to successfully procreate by mating with other hybrids, because of the irreversible accumulation of deleterious mutations in the clonally transmitted genome (Vorburger 2001, Guex et al. 2002, Vorburger et al. 2009). In general, hybridogenetic *P. esculentus* are reproductively dependent on their syntopic parental species in many populations and therefore are considered a sexual parasite (Graf and Polls Pelaz 1989, Joly 2001).

In some areas, hybrid *P. esculentus* populations with allopolyploid individuals exist and can reproduce and persist independently of the parental species. These populations usually consist of both diploid (LR) and triploid (LLR and LRR) individuals and occur both sympatrically with the two parental species and outside their range. In the latter case, they are considered all-hybrid polyploid populations and assigned to the EE-system type (Uzzell and Berger 1975, Graf and Polls Pelaz 1989). Triploid individuals usually produce haploid gametes containing the genome they have in double copy, while diploid females produce both haploid and diploid gametes (Vinogradov et al. 1991, Christiansen et al. 2005, Arioli 2007, Christiansen 2009, Christiansen and Reyer 2009). The fusion of diploid with haploid gametes result in viable triploid offspring, and diploid offspring are produced from the fusion of haploid gametes. Viable tetraploid individuals (type LLRR) do occur, but are very rare and do not seem to have a fitness advantage over diploid or triploid individuals (Arioli 2007, Jakob 2007, Christiansen 2009, Arioli et al. 2010, Jakob et al. 2010). From all-hybrid populations in Denmark and Sweden we know that triploid hybrids (genotype LLR and LRR) can recombine their double-copy genome (Arioli 2007, Christiansen and Reyer 2009), thus providing a mechanism of maintaining genetic diversity in the population and circumventing the danger of accumulating deleterious mutations.

The patchy distribution of polyploidy in *P. esculentus*

Despite the wide distribution of *P. esculentus* across Europe, the known distribution of polyploid populations is rather patchy, with areas where only non-polyploid hybrids and parental populations are found. Polyploid forms (i.e. normally triploids of the type LLR or LRR) have been found and studied in the following countries: Denmark (Fog 1994, Rybacki 1994, Christiansen et al. 2005), Sweden (Ebendal 1979, Ebendal and Uzzell 1982, Arioli et al. 2010, Jakob et al. 2010), Baltic States , Germany (Günther 1970, Günther 1974, Günther and Plötner 1990, Plötner and Klinkhardt 1992, Berger and Berger 1994), Poland (Berger 1988, Rybacki and Berger 2001, Czarniewska et al. 2011), Austria (Tunner and Heppich-Tunner 1991, Tunner 1994, Czarniewska et al. 2011), Czech Republic (chapter 3 of this thesis), Slovakia (Mikulíček and Kotlík 2001), Hungary (Tunner and Heppich-Tunner 1992, Brychta and Tunner 1994), Ukraine (Borkin et al. 2004, Mezhzherin et al. 2010) and Russia (Borkin et al. 2006). Several studies confirm the existence of at least two different reproductive modes in polyploid hybrids:

1) Triploid individuals of two types (LLR and LRR) and both sexes are formed through the fusion of haploid L or R gametes (produced by triploid hybrid of the LLR and LRR type, but also by diploid LR hybrids) and diploid, heterospecific LR gametes which are produced in varying quantities by LR females (Christiansen et al. 2005, Arioli 2007, Christiansen 2009, Christiansen and Reyer 2009, chapter 1 of this thesis).
2) Triploid, exclusively male hybrids of type LLR are formed through the fusion of haploid R gametes (provided by female diploid LR hybrids) and diploid LL sperm (produced by triploid LLR males) (Tunner 2000, Mikulíček and Kotlík 2001, chapter 3 of this thesis).

Objectives of this study

In Scandinavia, where polyploid all-hybrid populations are almost the only form of water frog populations, earlier studies have shown that the occurrence of polyploids is determined by the types of gametes being produced (Christiansen 2009) and that the genomic composition of populations varies with environmental factors (Arioli et al. 2010, Christiansen and Reyer 2011). However, since polyploidy in *P. esculentus* is not restricted to northern regions

(see above), we aimed to investigate the population genetic patterns within and among polyploid *P. esculentus* populations across a larger European area.

The objectives of our study were thus:

1. To examine **genetic diversity and genetic differentiation in water frogs over a large geographic scale and across a diverse selection of population types**. According to the post-glacial colonization theory we were especially interested whether populations differed in genetic diversity across areas along latitudinal and longitudinal clines. Alternatively, genetic diversity could be maintained by occasional crosses with parental species in populations where *P. esculentus* lives syntopically with the parental species. In this case, we would expect genetic diversity to be correlated with the presence of parental genotypes in the population.
2. To find **genetic structuring among polyploid populations** from different areas and genotypic composition using combined genetic information from microsatellites and mitochondrial DNA (mtDNA).

Material and methods

Sampling of genetic material

We sampled water frogs from 72 localities in 15 countries. The minimum distance between localities in this study was 2.63km, the maximum was 1863.5km. Short geographic distances between populations were the exception, as samples from nearby ponds that showed the same genotype distribution and similar pond features were usually pooled and considered belonging to the same population. Average geographic distance between populations was 727.8km ± 427.7km (1 S.D.). Most samples were collected by the authors. Samples from all Romanian, Bulgarian and Ukrainian populations, as well as some Hungarian populations, were kindly provided by the collaborators listed in the acknowledgements. All genetic samples were taken from tissue samples (toe clips). Genotype and ploidy of individuals were determined via microsatellite analysis. DNA extraction, PCR and electrophoresis followed protocols as in Christiansen and Reyer (2009). We

classified a population as “polyploid” when at least one polyploid hybrid individual was found in the sample and verified through multiple microsatellite markers. Populations were classified as “diploid” when only diploid hybrids were found in the sample and when the absence of polyploid forms in this area was not contradicted by local colleagues or by the recent literature. Among the populations in our study, this classification was unambiguous, since whenever we detected polyploid individuals in a population, they were always present in numbers >1.

Microsatellite markers

We used a set of 18 microsatellite DNA markers run in four primer mixes:

• Primer Mix 1A - CA1b6, Ga1a19 redesigned (Arioli et al. 2010), RlCA1b5, RlCA5 (Garner et al. 2000), Rrid064A (Christiansen and Reyer 2009)

• Primer Mix 1B - Re2CAGA3 (Arioli et al. 2010), Res16, Res20 (Zeisset et al. 2000), RlCA2a34 (Christiansen and Reyer 2009)

• Primer Mix 2A - ReGA1a23, Rrid169A, Rrid059A redesigned (Christiansen and Reyer 2009), Res22 (Zeisset et al. 2000), Rrid013A (Hotz et al. 2001)

• Primer Mix 2B (PM2B): Re1CAGA10 (Arioli et al. 2010), RlCA18 (Garner et al. 2000), RlCA1a27, Rrid135A (Christiansen and Reyer 2009).

Details on PCR protocols are given by Christiansen (2009) and Christiansen and Reyer (2009, 2011). We ran PCR products on an ABI 3730 Avant capillary sequencer (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA) with internal size standard (GeneScan-500 LIZ).

Multilocus genotypes were established from the allele data in a step-wise procedure. First, alleles were scored without knowledge of locality or genotype (LL, LLR, LR, LLRR, LRR and RR). Then, with a combination of field notes describing the supposed taxon of the individual based on morphological characters and prior expectations of L and R specificity from previous studies (Christiansen and Reyer 2009, 2011), consensus genotypes were determined for all individuals. On the basis of these consensus genotypes, genome specificity was assigned to previously unknown alleles. The ploidy of the consensus genotypes was verified by analyses of dosage effects at four loci (Res16, Ga1a19, RlCA1b5 and CA1b6), following the method described in (Christiansen 2005). Within each locus, peak heights were obtained using

program Genemapper 3.7 (Applied Biosystems). Genotypes and peak sizes were manually proof-read, and plots of log (height1/height2) were drawn for all pairwise combinations of alleles in the entire data set. These plots were visually examined for groups of individuals corresponding to 2:1, 1:1 and 1:2 allele ratios. Depending on the genome specificity of the alleles, these ratios could be translated into LL, LLR, LR, LRR, and RR genotypes (LLL, LLLR, LRRR and RRR were not found). LLRR tetraploids might appear as LR at some dosage effect loci, but are usually revealed by heterozygosity for L and R alleles at other loci. Due to the low occurrence of LLRR in natural populations, the danger of misclassifying an LLRR individual as LR is very low (Christiansen 2009, Arioli et al. 2010).

Loci in conflict with the consensus genotype were examined again or rerun in PCR and fragment analysis. Loci that were still incongruent with the consensus genotype after this extra round of evaluation were treated in one of three ways. When only few frogs from the same locality (populations with $n < 15$: 1-2 individuals; populations with $n > 15$: up to 3 individuals) showed the same kind of incongruence at the same locus, this locus was coded as missing data, as the problem was not quantitatively important. When more frogs were concerned, the problem could usually be assigned to either allele inspecificity or null alleles. In cases of allele inspecificity, single alleles were re-assigned to either the L or R genome to fit the consensus genotype. Some loci turned out problematic for one or both genomes (in L: RlCA5, Res20, Rrid013A; in R: RlCA2a34, Rrid169A; and in both L and R: ReCaga10), since missing values accumulated in high numbers and in a non-random pattern despite multiple re-runs of the affected samples, which indicated a systematic failure of sample batches rather than the occurrence of true null alleles. Especially populations from Ukraine and Romania were affected by systematic failure of amplification. Therefore, existing values in these loci were used only for confirmation of the consensus genotypes, but in any subsequent analyses the whole locus was omitted for all populations. Also, one marker (Rrid059A) turned out almost monomorphic in the L genome by yielding only two alleles across all samples. To avoid strong differences in polymorphy among markers, the affected locus was omitted from the L genome data set.

This procedure yielded a total number of 8 markers for the L genome and 10 markers for the R genome data set (Table 1). To avoid bias estimates of allele frequencies, we carefully checked the remaining loci data for the occurrence of true null alleles. Null alleles were either directly detectable in the four dosage effect loci (in the case of a null allele, the actual dosage ratio did not fit the ratio expected by the consensus genotype), or by non-amplification of non-dosage markers in hemizygous individuals. For example, a null allele occurring at a non-dosage L-specific locus would not be detectable in a heterozygous LLR or LL frog, but unmasked in homozygous diploid (LL) stages as in LLR or LL frogs, and also in LR and LRR frogs that carry only one copy of the L genome. If only few individuals per population were affected (see above), the locus in these individuals was coded as missing data. According to this protocol, we identified null alleles in three of the markers in more than 2-3 individuals in a total of three populations. These null alleles were re-coded as real alleles.

For population genetic analyses, the L and R genomes were split into two independent data sets and analyzed separately. Since many of the sampled populations consisted of a mix of hybrid and non-hybrid frogs (i.e. *P. lessonae* and *P. ridibundus* of genotypes LL and RR), and since homospecific genotypes can only occur in one of the two data sets, some populations differed in sample numbers between the two data sets (see Table 2). Due to the mix of haploid and diploid genomes in our full data set, we restricted further analyses on genetic differentiation and structuring to allele-frequency-based methods rather than methods based on observed heterozygosity. As a measurement for genetic diversity we used H_e (expected heterozygosity according to Nei, 1978) calculated by program SPAGeDi 1.3 (Hardy and Vekemans 2002). SPAGeDi can handle a mix of haploid and diploid data and was further used to calculate Nei's D, F_{ST} and geographic distance matrices among populations. Mantel tests between genetic distance and geographic distance matrices were calculated with program zt (Bonnet and Van de Peer 2002). Pairwise geographic distances were calculated from GPS coordinates using *Geographic Distance Matrix Generator version 1.2.3* (Ersts Internet). Cluster analyses for L and R genomes in polyploid populations were performed on the basis of pairwise Nei distances, using the

Euclidian distance metric and the single linkage aggregation criteria implemented in the software PermutMatrix Version 1.9.3, (Caraux and Pinloche 2005).

Mitochondrial DNA sequence analysis

We analyzed mtDNA sequences of 1175 individuals from 105 localities that represented the range of geographic and population type variation. Since we later included some sequences from previous studies, populations were not entirely identical with the ones listed in Table 2. We sequenced two genes, ND2 (1038 bp) and ND3 (340 bp), which have previously been used for phylogenetic and phylogeographic investigations (e.g. Plötner 2005, Arioli 2007, Plötner et al. 2008, Akin et al. 2010). DNA extraction, PCR and sequencing were conducted following closely the protocol described in Plötner et al. (2008). For initial alignments of mitochondrial DNA sequence fragments we used the inbuilt alignment tool Clustal W in program MEGA version 5.05 (Tamura et al. 2011). Subsequently, the alignment was improved manually. For sequence statistics we used MEGA 5.05. For a maximum likelihood estimation of haplotype genealogies, we calculated the best correction model for genetic distances based on complete ND2 and ND3 sequences in program jmodel test version 0.1.1. For maximum likelihood inference, the Hasegawa-Kishino-Yano-85 model (Hasegawa et al. 1985) with gamma-distributed rate heterogeneity and correction for invariant characters (HKY + G + I) yielded the best result. Phylogenetic trees were calculated and visualized in program MEGA version 5.05. Branch support was evaluated by bootstrapping with 1000 replicates.

Results

Population type and genotype distribution

Based on microsatellite profiles, we genotyped and analyzed a total of 2062 frogs from 72 localities. The most numerous taxon was *P. esculentus* with 63 % of all genotyped individuals, followed by *P. ridibundus* (25.5 %) and *P. lessonae* (11.5 %). *P. esculentus* occurred in 50 localities (69 %), *P. ridibundus* in 40 (56 %), and *P. lessonae* in 27 localities (38%). In our sample, we found all-*ridibundus* populations (n=20, 26% of localities) almost

exclusively south of 48° latitude and east of 16° l ongitude (Figure 1), especially in the proximity of the numerous tributaries to the Danube river and the Black Sea. One all-*ridibundus* population, however, was situated quite remotely from the rest in the Baltic area (Jumala). We found only two all-*lessonae* populations (Uppsala and Laeva, 2.6% of all samples), both of which were among the northernmost populations in the sample. The remaining populations included hybrid *P. esculentus* of various ploidies levels. Of these, 26 (36% of total) were classified as diploid, and 24 (33%) were classified as polyploid populations. Fourteen (17 %) localities included both types of triploid hybrids, LLR and LRR. In another eight populations (11%), LLR was the only type of triploid hybrid, whereas five populations (7%) included only the LRR genotype. All triploid individuals co-occurred with diploid LR hybrids, and in half of the cases polyploid hybrids additionally co-occurred with either *P. ridibundus* or *P. lessonae*. Only in two populations (Sanie in Poland; Buchach in Ukraine), diploid and polyploid hybrids occurred together with both parental genotypes RR and LL. Within 54 % of polyploid populations (13/24), no parental genotypes were found. Only four tetraploids of the genotype LLRR were detected in three populations (Bornholm in Denmark, Untermassfeld in Germany; Sanie in Poland) and together made up 0.2 % of all samples.

Effects of geographic location and genotypic composition on genetic diversity

The ten loci used for analysis in the R genome yielded 220 alleles (range: 6-41 per locus) in total for a sample size of 1807 individuals from 66 populations. The eight loci used for analysis in the L genome yielded a total of 100 alleles (range: 5-22 per locus) across the entire sample of 1506 individuals from 50 populations. Gene diversity was generally lower in the L genome than in the R genome (mean H_{eL}: 0.301; mean H_{eR}: 0.439). H_{eL} was also lower than H_{eR} in every marker that amplifies both genomes (Table 1). We investigated whether the specific geographic locality and genotype composition influences genetic diversity in the R and L genome. General linear models on genetic diversity (H_{eL} and H_{eR}) incorporating geographic latitude, longitude, percent of parental species (LL for H_{eL}, RR for H_{eR}) and percent of polyploid individuals in the population yielded a strong overall effect

of this set of variables on genetic variation in both genomes (H_{eL}: multiple R^2 = 0.51; H_{eR}: multiple R^2 = 0.75).

Latitude, percentage of parental species (RR), frequency of polyploids – and to a lesser extent longitude – gave significant results on H_{eR} (Table 3). Genetic diversity in the L genome (H_{eL}) was less affected by longitude and not by the percentage of polyploid individuals (Table 3). In both genomes, diversity declined with increasing latitude, as indicated by the negative regression coefficient (Table 3, visualized in Figures 2 and 3). In the R genome, diversity increased with longitude (Figure 3) and both the percentage of parental (RR) and polyploid genotypes in the population (Table 3). Percentage of parental genotypes (LL) also increased diversity in the L genome, but the percentage of polyploids did not show a significant effect (Table 3). In contrast to H_{eR}, H_{eL} did not show a linear increase with longitude, but rather yielded a more quadratic relationship, where diversity increases to a degree of longitude of approx. 25° and then decreases again further east (Figure 2). The highest diversity in the L genome was found between 45° and 50° latitude and 16° and 25° longitude, which is also the geographic band where most L-E populations (diploid hybrids in sympatry with *P. lessonae*) occurred (Table 2 and Figure 2). The influence of geography on genetic diversity was also evident in the three northernmost parental populations, where we found comparatively low values for genetic diversity: the remote all-*ridibundus* population in the Baltic area (Jumala) had the second lowest value for genetic diversity among all pure *P. ridibundus* populations (H_{eR} = 0.48, average across all-ridibundus populations: H_{eR} = 0.69), and genetic diversity in the two all-*lessonae* populations (Laeva: H_{eL} = 0.28, Uppsala: H_{eL} = 0.19) was also below the overall average (see above).

F_{ST} and isolation by distance

For the entire sample, calculation of global F_{ST} yielded 0.349 for the L genome and 0.294 for the R genome, thus attributing 34.9% of the genetic variation in the L genome and 29.4% of the genetic variation in the R genome to inter-population differences.

When we tested for isolation by distance across all populations, we found genetic distance (given as Nei's D_S) to increase strongly with geographic

distance between populations in both genomes (one-tailed Mantel test: L: r = 0.63, p = 0.00001; R: r = 0.65, p = 0.00001; Figure 4). The same tests on F_{ST}-values between populations yielded similar results, yet the effect was smaller (one-tailed Mantel test: L: r = 0.22, p = 0.00001; R: r = 0.35, p = 0.00001). When we only compared genetic distance matrices (given as Nei's D_S) of polyploid populations, we found that most populations showed moderate to great genetic differentiation to each other in both genomes, as indicated by the darker color shades in Table 4. The populations in Table 4 were sorted by proximity in geographic longitude, and populations with little genetic differentiation (lighter shades) were usually from geographically close localities. Mantel tests supported isolation by distance (Nei's D versus geographic distance) between the 24 polyploid populations (L: r = 0.54, p = 0.0003; R: r = 0.43, p = 0.0035), which indicate that polyploid populations generally behave in the same way as the whole set of populations we sampled.

Genetic diversity and population structure: microsatellites

Based on genetic distance (Nei's D), we performed separate cluster analyses for the L and R genome among the 24 polyploid populations. Details of the cluster tree of the L genome (Figure 5) revealed two major clusters that were more similar to each other than to the rest of the populations. These clusters comprise north-western populations (orange and pink numbers Figure 7a). Furthermore, three smaller clusters were identified, plus three populations that were associated, but not clearly form a tight cluster. Figure 7a illustrates the genetic clusters on the European map: the main clusters (orange and pink) in Northern and Central Europe, a rather spread-out cluster in Central Poland and Ukraine north of the Carpathian mountains (green), a small cluster in Slovakia (blue) and one genetically and geographically distinct cluster in Eastern Ukraine (red) (Figure 7a). In general, differentiation between clusters (except for orange/pink) in the L genome was fairly high. Two island populations in the Baltic Sea, Fehmarn (13) and Bornholm (4) were remotely associated with the central and east-central clusters, but shared low similarity. The population of Shatsk (50) in Northern Ukraine also fell in between clusters. Differentiation in the R genome was somewhat smaller, with the

result that we could identify only three clusters (Figure 6). On the map (Figure 7b), the main cluster (pink) includes all population in the northern and central parts up to the Ukrainian border in the East. Two populations close to large rivers in Western and Northern Ukraine form another cluster (green). The last cluster includes the two most eastern Ukrainian populations (red), which mirrors the distinct red cluster in the L genome map (Figure 7a).

Genetic diversity and structure: mtDNA

We sequenced 1175 samples from 102 populations, which yielded a total of 75 haplotypes. The *lessonae*-specific ND2 + ND3 sequences exhibited 30 variable sites (25 in ND2 and 5 in ND3), which resulted in 40 haplotypes (Table 5). Nucleotide diversity (Pi) among *ridibundus* haplotypes was 0.0031 ± 0.0007. Overall, mean genetic distance was 0.31% among *lessonae* haplotypes (range: 0.82% to 0.07%). *Ridibundus*-specific ND2 + ND3 sequences exhibited 35 variable sites (29 in ND2 and 6 in ND3), which resulted in 32 haplotypes (Table 5). Among these, nucleotide diversity (Pi) was 0.0068 ± 0.0012, and overall mean genetic distance amounted to 0.67% (range: 1.57% to 0.07%). Most samples carried haplotypes that were *lessonae*- (n=806, 68.6% of samples) or *ridibundus*-specific (n=343, 30.0%), but we also found two haplotypes of mtDNA specific to *P. bergeri* (n=15, 1.3%), and one haplotype that could be assigned to an Anatolian clade of *P.* cf. *bedriagae* (n=1). Figure 8 gives an overview of the distribution of *lessonae*- (Figure 8a) and *ridibundus*-specific (Figure 8b) mtDNA found in the selected populations with respect to assigned population type. A phylogenetic tree analysis yielded significant differentiation between *lessonae*- , *ridibundus*- , *bergeri*- and the Anatolian cf. *bedriagae*-type of mtDNA (Figure 9). Within the *lessonae*-group, differentiation was generally low, and only two clusters were weakly, yet not significantly, supported. Polyploid individuals (LLR, LRR) were found in both clusters with a total of 16 haplotypes.

We did not recognize an explicit geographic pattern in the distribution of *lessonae*-haplotypes (Figure 8a). Eight *lessonae*-haplotypes (from both clusters) were only found in *P. lessonae* individuals, and seven haplotypes from cluster les-1 were found also in *P. ridibundus* – apart from their occurrence in hybrids and *P. lessonae*. The *ridibundus*-group yielded

significant differentiation into two clusters, although the degree of differentiation was also low (Figure 9). Cluster rid-1 yielded one very common haplotype that occurs across several countries in both *P. esculentus* and *P. ridibundus* (Figure 8b). The clusters contained eight other haplotypes that occurred in *P. ridibundus* specimen from Hungary, Slovakia, Croatia, and Bulgaria, as well as in two LR individuals from a Ukrainian hybrid population. In the second cluster, haplotypes found exclusively in *P. ridibundus* dominated, but compared to the first cluster, more hybrid carriers of *ridibundus*-haplotypes were found. Within cluster two, also one very common *ridibundus*-haplotype (R6) was detected – for the first time - in polyploid individuals. Despite the wide distribution of R6 covering at least seven countries (Table 5), but has not been found in polyploid individuals anywhere else than in five LRR individuals from the population of Gaidary, Ukraine.

Discussion

Genetic diversity as a function of latitude, longitude and occurrence of parental species

Our study shows substantial geographical overlap among the different population types. Yet, some distribution pattern can be recognized (Figure 1). Polyploid hybrid populations are rather common in north-central Europe up to southern Sweden but do not occur further south than to the rims of the Carpathian mountains. Diploid hybrid populations were common in central Europe, pure *P. ridibundus* dominated south of the Alps and Carpathians and pure populations of *P. lessonae* were found only at two remote localities in Sweden and Latvia. This pattern widely confirms the distribution of the continental water frog species as reviewed and described by Plötner (2005).

The impression that diploid hybrid populations often occur close to mountain ranges like the Alps, the Carpathians or the Harz and pure *P. ridibundus* populations are more numerous close to large rivers and coastal areas of the Black Sea may be more a reflection of where we sampled, rather than of the actual habitat preferences. The same methodological explanation may hold for the scarcity of polyploid populations in the Eastern Ukraine

where we had samples from only a few localities, but polyploids are known to be very common (Borkin et al. 2004).

The high density of polyploid populations across a defined area stretching from Southern Sweden to Germany and into Poland is representative (Rybacki and Berger 2001, Christiansen et al. 2005, Arioli et al. 2010, Christiansen and Reyer 2011), and so is their low density in the east-central part of Europe, just slightly north of the gap between the Alps and the Carpathian range. The three localities in the Czech Republic (Borovec) and Slovakia (Šajdíkove Humence, Kozi Chrbát) that we included in our sample basically cover the area where triploids exist (Mikulíček and Kotlík 2001, chapter 3 of this thesis).

Genetic diversity in our sample was generally lower in the L genome than in the R genome. This is probably attributable to the dominance of all-*ridibundus* populations (20 versus 2 all-*lessonae*) among the pure parental populations in our sample. Pure populations of *P. ridibundus* are very common in Southeastern Europe and showed the highest genetic diversity values (H_{eR}). Pure *P. lessonae* populations are rare (Plötner 2005) and seems to occur only in the marginal northernmost parts of the pool frog's distribution area, where they show low genetic variation (Sjögren 1991, Sjögren-Gulve and Berg 1999). In both the L- and the R-genomes of *P. esculentus*, genetic diversity decrease with higher latitude despite differences in distribution and frequency of sympatry between hybrids and parental species. According to the postglacial refuge theory, rapid northwards expansion following interglacials often resulted in low genetic diversity caused by repeated founder effects that led to a loss of alleles and heterozygosity (Hewitt 1999). Rapid long-distance northward dispersal at low altitudes and along river valleys during warm climate periods appear likely for water frogs (Zeisset and Beebee 2001). In the case of such rapid expansions, few long-distance dispersers can lead to successful founding events that inhibit the establishment of later arriving genotypes. This phenomenon is also known as high-density blocking (Waters et al. 2013). However, in the case of the polyploid all-hybrid populations in Northern and North-Central Europe, we can assume that their success is also the result of a continuous competitive advantage over their parental species. Christiansen (2009) very elegantly demonstrated in a data-

based equilibrium model that both parental genotypes would dominate (in the case of RR) or even drive all-hybrid populations to extinction (in the case of LL) after less than 40 generations if their survival rates were higher than those of the hybrids.

Since we know that at least *P. lessonae* succeeded in expanding its postglacial range to high latitudes (Sjögren 1991, Sjögren-Gulve and Berg 1999, Zeisset and Beebee 2001), the dominance of *P. esculentus* in Northern and North-Central Europe thus indicates some continuous competitive advantages during the colonization of the areas formerly covered by glaciers. One competitive advantage in Northern regions with shorter and cooler summers could be that *P. esculentus* hybrids develop faster and perform better at colder temperatures during their larval stages than parental genotypes (Negovetic et al. 2001, Pruvost et al. 2013). Since hybrid water frog females have higher fecundity than parental females (Berger and Uzzell 1980), hybrid offspring can quickly outnumber parental genotypes when the conditions favor the hybrids' survival. Sometimes this is compensated by low reproductive success of hybrid males in mixed populations with *P. lessonae* (Abt and Reyer 1993), but in polyploid populations of the EE-system, hybrid-hybrid pairings result in more viable offspring than interhybrid matings in hybridogenetic (non-polyploid) systems, because of the polyploids' ability to recombine homologous genomes (Christiansen and Reyer 2009). Another advantage might lie in the evolution of different hybrid lineages (Hotz et al. 2008) caused by repeated events of primary hybridization in the suture zone of *P. lessonae* and *P. ridibundus*, which later allowed for local selection among these hybrid haplotypes. This may have resulted in some *P. esculentus* genotypes that are very common and can occupy a broad ecological range, but also in genotypes that have small geographical ranges and suggest more ecological specialization (Pagano et al. 2008). Hybrid *P. esculentus* even appear more resilient against virulent pathogens than non-hybrid water frogs (Daum et al. 2012).

In sum, the success of polyploid all-hybrid populations across large connected distribution areas as the range of the EE-system of north-central Europe is probably the result of a combination of competitive advantages and reproductive independence from the parental species. In most regions that are

further south, diploid hybrid or pure parental (RR) systems prevail, which indicates that polyploid hybrids are no longer at a general advantage, but manage to exist only in sympatry with *P. lessonae* or *P. ridibundus*, or at least in patchy “enclaves” where local hybrid haplotypes perform better than non-hybrids.

Genetic distance between polyploid populations

We found that genetic differentiation among polyploid populations was generally high. On the one hand, this can be attributed to isolation by distance, which our data supported for all water frog populations, irrespective of their hybrid status or ploidy. On the other hand, polyploidy could theoretically have added to the degree of genetic differentiation. In plants, polyploidy can induce novel gene combinations through homologous recombination (Gaeta and Pires 2009) which can be advantageous, provided that stable chromosomal inheritance is established in the population through natural selection. In order to observe strong differentiation among populations caused by polyploidization, this would require at least several of such events and subsequent isolation between polyploid populations.

We performed hierarchical clustering analyses based on microsatellite data to identify a structure of genetic similarity among polyploid populations. We could gather a fairly congruent pattern from separate cluster analyses of genetic distances in the L and R genome. In general, differentiation and structuring in the R genome was weaker than in the L genome, resulting in fewer clusters. Most clusters grouped populations, that were probably connected by geographic proximity or common routes of colonization, e.g. along river systems. Yet, we did find some interesting exceptions. Both analyses identified at least one (R genome) or two (L genome) uniform clusters in north-central Europe that covered most populations in Sweden, Denmark, Germany and Poland. As an outlier in the L genome, the population on the island of Bornholm formed a separate cluster, which it surprisingly shared with a population near Kiev, Ukraine, although the F_{ST} value between the two populations is high (> 0.25). A polyploid Czech population (Borovec) belonged to the north-central cluster, while the two close by Slovak populations (Šajdíkove Humence and Kozi Chrbát) form a separate cluster of

their own. This is remarkable, since these three populations share a particular breeding system that differs from the north-central EE-system insofar, as triploids only occur as LLR, are male-only and are produced through the fusion of diploid homospecific sperm (LL) with haploid eggs (R) rather than through haploid sperm (L or R) and heterospecific diploid (LR) eggs (Mikulíček and Kotlík 2001, chapter 3 of this thesis). However, other than the two Slovak populations, Borovec is connected to the Odra river system and apart from this particular polyploid haplotype hosts other hybrid types common in Poland and Eastern Germany. Two more distinct L genome clusters were formed by two pairs of populations in Ukraine. Interestingly, analysis of the R genome assigned one of the Ukrainian L-clusters (Shatsk and Buchach) to two other clusters, namely Shatsk to the huge R cluster that includes all populations west of the Carpathians, and Buchach to Baturin, which is in the north of Ukraine close to the city of Kiev. This indicates that the genetic influence from other populations can differ between the L and R genome. However, clustering of the two remaining populations in East Ukraine (Gaidary and Zhvotneve) was the same in both analyses, illustrating that these two populations are genetically set off from other polyploid populations in both parts of their hybrid genome. The question, whether genetic differentiation among reproductively independent hybrid clades will eventually lead to speciation among polyploid hybrids, remains still open. Speciation would require some degree of reproductive and/or ecological isolation. According to recent studies, there is little reproductive isolation between water frog populations from different population systems and regions in North and Central Europe (chapter 1 of this thesis). Crossing experiments between frogs of different breeding systems including individuals from geographically distant populations (e.g. from the EE-system and from Eastern Ukraine) would thus further expand our knowledge about the evolutionary potential of genetically differentiated polyploid water frog populations.

Patterns of mtDNA haplotypes

Plötner et al. (2008) previously investigated patterns of mtDNA transfer in European water frogs based on a large sample covering an area comparable to this study. They found that while introgression of *ridibundus*-type mtDNA

was never found in *P. lessonae*, occurrence of *P. ridibundus* specimens carrying introgressed *lessonae*-specific mtDNA was common in Central Europe and closely correlated with sympatry of *P. esculentus*. In Eastern and Southeastern Europe, *P. ridibundus* exclusively carries *ridibundus*-specific mtDNA, irrespective of the occurrence of *P. esculentus* or *P. lessonae*. Our results confirm most of these findings, like the absence of rid-type mtDNA in *P. lessonae* and the common presence of introgressed *les*-type mtDNA in Central European *P. ridibundus*. Interestingly, hybrids appear to mirror this pattern. While *P. esculentus* carries only *lessonae-specific* mtDNA in areas where presently no primary hybridization can occur because of the absence of *P. ridibundus* (e.g. Switzerland), diploid hybrids can carry both types in areas where both parental species occur and primary hybridization is possible (Spolsky and Uzzell 1986). This was also true in our study.

According to Plötner et al. (2008), triploid hybrids from polyploid populations always had les-type mtDNA without exception. For most polyploid individuals investigated in this study, our data confirm this. However, we identified one *ridibundus*-haplotype in five polyploid LRR frogs from a population in Eastern Ukraine. Borkin et al. (2004, 2006) observed that polyploidy (LLR and LRR, very rarely tetraploidy) in *P. esculentus* occurs at high quantities and across different population types (including mixed systems with *P. lessonae* and *P. ridibundus*, or both) in Ukraine and Russia, especially along the Donets River, a large fluvial area in Eastern Ukraine. It appears that these eastern populations of polyploid hybrids are similarly wide-spread and successful as the hybrids of the north-central European EE-system, although we found that they are genetically different. Since *P. esculentus* is the result of multiple hybridization events and its hybridogenetic lineages yield a diversity of hemiclones (Hotz et al. 2008, Pagano et al. 2008), it is very probable that polyploid lineages evolved several times independently, which would mean that the polyploid population clusters we identified in our study have different origins. For other amphibian taxa, multiple origins of polyploid lineages are well documented (Ptacek et al. 1994, Holloway et al. 2006), thus giving support to the hypothesis that several hybridization events and regular interaction of parental genotypes, rather than one unique “accident”, may be

the normal route leading to the successful establishment of allopolyploidy (Dowling and Secor 1997).

Introgression beyond breeding systems

Apart from the existence of different genetic clusters among polyploid populations, we also found mitochondrial introgression from water frog species other than *P. lessonae* and *P. ridibundus* into *P. esculentus* population systems. MtDNA typical for the Italian pool frog *P. bergeri* has been previously reported to occur in *P. lessonae* and diploid *P. esculentus* from Switzerland and Southern Germany (Hotz et al. 1992, Plötner et al. 2008). In our study, *bergeri*-type mtDNA was found in the three westernmost populations, two of them being diploid populations (Hellberg, Switzerland, and Herzberg, Central Germany) and one a polyploid population containing few RR individuals (Untermassfeld, Central Germany). Whereas in the two diploid populations *bergeri*-specific mtDNA was carried both by *P. lessonae* and LR hybrids, in Untermassfeld (where *P. lessonae* is probably absent) we found *bergeri*-specific mtDNA only in diploid hybrids and – for the first time - in one triploid LRR female. Our microsatellite data show that the Untermassfeld population is - although not more genetically diverse than other German populations - very diverse in terms of ploidy types and types of mtDNA. The Untermassfeld population contains both types of triploids, some *ridibundus* genotypes and three types of mtDNA: *lessonae, ridibundus* and *bergeri*. While the *lessonae*-type is typical for north-central all-hybrid populations, *ridibundus*-haplotypes are rare in Central European hybrid populations and are much more common further East. Finally, the *bergeri*-type has been documented only in Swiss and German LE-systems. Since Untermassfeld lies right in the center of Germany, it is possible that frogs from different breeding systems (and possibly different colonization routes) have encountered there and mutually exchanged parts of their DNA, which passed through various types of hybrids. Whether these frogs got there by themselves (e.g. along major rivers) or were brought there recently by humans (Untermassfeld is a fish breeding pond, where amphibian larvae could have brought in with fish larvae from other areas), remains to be investigated.

At the easternmost edge of our range of sampling localities we found another novel record of heterospecific introgression of mtDNA into *P. esculentus*. For the first time, a diploid *P. esculentus* hybrid (as identified by microsatellite genotype) from Gaidary Iskov (52, Southeastern Ukraine) was identified to carry an Anatolian-type cf. *bedriagae*-specific mtDNA. A combination like this could come about when a hybrid *P. esculentus* male mated with a female *P.* cf. *bedriagae,* or when a *P. esculentus* male mated with a *P. ridibundus* female carrying cf. *bedriagae*-specific mtDNA as a heritage from an earlier hybridization between *P. ridibundus* and *P.* cf. *bedriagae*. The first scenario should be less likely, since the distribution area of *P.* cf. *bedriagae* ranges from Western Anatolia to the Caspian Sea (Akin et al. 2010). Therefore, we favor the scenario of a previous hybridization between *P. ridibundus* and *P.* cf. *bedriagae,* which could have taken place before *P. ridibundus* extended its range from the Black Sea northwards, since there is evidence for migration of water frogs from Anatolia into Europe and hybridization between Anatolian and European individuals, for instance in Eastern Greece (Hotz et al. 2013). All in all, we found evidence that the allopolyploid hybrid *P. esculentus* not only incorporated genetic information from its two original parental species, but apparently succeeded to extend its genetic heritage to other water frog species living close to the respective distribution borders of *P. lessonae* (proximity to contact zone with *P. bergeri*, in our study: Switzerland and Germany) and *P. ridibundus* (the transition zones to Anatolian water frogs in Eastern Greece and west of the Caspian Sea).

Conclusions

We suggest a colonization scenario according to which hybridogenetic *P. esculentus* were repeatedly created through primary hybridization between *P. lessonae* and hybridogenesis-inducing *P. ridibundus* genotypes in a postglacial suture zone southeast of the Alps and Carpathians. From there they probably expanded to Central and Northern Europe, following the northwestwards branch of a bifurcating colonization route that has been suggested for *P. lessonae* (Zeisset and Beebee 2001) and other European amphibians (Hewitt 1999, Stöck et al. 2012). Along the migration route, the

first polyploid hybrids could have emerged through pairings between diploid hybrid males and diploid hybrid females which, as a novelty, produced diploid eggs as it is characteristic of LR females from EE- and LE-system populations (e.g. Berger and Uzzell 1980, Graf and Polls Pelaz 1989, Arioli 2007, Czarniewska et al. 2011). Colder climates during the reproductive season could have played a role in inducing polyploidy during gametogenesis (Kawamura 1984, Kondo and Kashiwagi 2004). Other groups of hybrids probably extended their range from the Black Sea area towards the area of today's Ukraine, where strong genetic differentiation and novel introgression patterns of mtDNA suggest at least one more separate system of polyploid *P. esculentus* that even might not share the same phylogenetic origin than the other polyploid populations in our study.

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Tables

Table 1: Microsatellite allele numbers and genetic diversity for L (H_{eL}) and R genomes (H_{eR}).

Locus	L alleles	R alleles	both L + R	HeL	HeR
CA1b6	3	18	2	0.486	0.730
RICA1b5	7	8	0	0.099	0.275
Ga1a19redesigned	4	29	1	0.038	0.652
Res16	2	9	4	0.086	0.468
Rrid064A		19			0.733
Re2Caga3		41			0.900
Res22		27			0.644
Rrid013A		6			0.249
Rrid059Aredesigned		29			0.645
Rrid135A		27			0.718
RICA2a34	18			0.734	
ReGa1a23	22			0.860	
Ca1A27	15			0.827	
RICA18	22			0.687	

Table 2: Geographic coordinates, sample sizes and genotypic composition of water frog populations sampled for microsatellite analysis. Classification was based on microsatellite results and was complemented by additional information on the population through personal observation (e.g. on presence of parental genotypes even though none were captured for sampling).

Nr.	Country	Population	Latitude	Longitude	n L	n R	N	% LR	% RR	% LL	%LLR	%LRR	%LLRR	HeL	HeR	classification
1	Sweden	Uppsala	60°33'24.52"N	17°51'46.04"E	20		20	0	0	100.00	0	0	0	0.19		all-lessonae
2	Sweden	Östergötland	58°5'38.59"N	16°22'28.68"E	23	12	23	52.17	0	47.83	0	0	0	0.12	0.00	diploid
3	Sweden	Genarp	55°36'34.00"N	13°23'19.00"E	63	80	80	70.00	0	0	11.25	18.75	0	0.15	0.09	polyploid
4	Denmark	Bornholm	55°7'28.72"N	15°8'58.57"E	32	38	38	47.37	13.16	0	7.89	26.32	5.26	0.13	0.41	poly
5	Estonia	Laeva	58°25'41.99"N	26°19'7.99"E	20		20	0	0	100.00	0	0	0	0.29		all-lessonae
6	Latvia	Stickli	57°19'41.00"N	22°15'21.99"E	20		20	30.00	0	70.00	0	0	0	0.31		diploid
7	Latvia	Jumala	56°59'36.99"N	25°55'22.00"E		14	14	0	100.00	0	0	0	0		0.48	all-ridbundus
8	Lithuania	Baltoji Voke	54°28'44.00"N	25°7'59.00"E	24	12	24	50.00	0	50.00	0	0	0	0.32	0.31	diploid
9	Poland	Kolczewo	53°57' 54.0"N	14°36'34.10"E	7	7	7	71.43	0	0	14.29	14.29	0	0.05	0.32	polyploid
10	Poland	Wysoka	53°49'53.47"N	14°51'51.53"E	37	37	37	54.05	0	0	35.14	10.81	0	0.21	0.44	polyploid
11	Poland	Sanie	51°25'38.10"N	16°56'57.84"E	54	48	61	62.30	8.20	21.31	1.64	3.28	3.28	0.39	0.29	polyploid
12	Poland	Krakow	50°5'3.60"N	19°50'26.46"E		12	12	0	100.00	0	0	0	0		0.51	all-ridbundus
13	Germany	Fehmarn	54°31'9.984"N	11°3'13.96"E	26	26	26	65.38	0	0	34.62	0	0	0.17	0.24	polyploid
14	Germany	Rügen	54°25'1.44"N	13°23'45.39"E	16	16	16	56.25	0	0	43.75	0	0	0.26	0.25	polyploid
15	Germany	Klützer Winkel	53°59'23.34"N	11°0'37.02"E	26	26	26	30.77	0	0	38.46	30.77	0	0.25	0.33	polyploid
16	Germany	Usedom	53°52'44.82"N	14°8'20.58"E	21	21	21	33.33	0	0	57.14	9.52	0	0.27	0.49	polyploid
17	Germany	Grammentin	53°45'26.72"N	12°53'41.04"E	19	19	19	21.05	0	0	36.84	42.11	0	0.22	0.29	polyploid
18	Germany	Schönermark	52°54'7.08"N	12°19'15.50"E	79	78	79	46.84	0	1.27	15.19	36.71	0	0.32	0.39	polyploid
19	Germany	Teschendorf	52°51'53.03"N	13°8'40.38"E	34	33	34	17.65	0	2.94	76.47	2.94	0	0.29	0.32	polyploid
20	Germany	Altenhausen	52°16'40.0"N	11°15'15.01"E	29	26	29	27.59	0	10.34	58.62	3.45	0	0.29	0.49	polyploid
21	Germany	Cottbus	51°46'24.30"N	14°21'19.14"E	42	49	49	40.82	14.29	0	16.33	28.57	0	0.11	0.45	polyploid
22	Germany	Herzberg	51°37'36.66"N	10°21'15.06"E	35	50	60	41.67	41.67	16.67	0	0	0	0.44	0.40	diploid
23	Germany	Döbern	51°36'38.22"N	14°36'15.60"E	63	63	63	50.79	0	0	15.87	33.33	0	0.20	0.28	polyploid
24	Germany	Untermassfeld	50°32'21.06"N	10°24'28.44"E	39	44	44	61.36	11.36	0	0	25.00	2.27	0.38	0.43	polyploid
25	Czech Rep.	Břidličná	49°55'00.40"N	17°21'39.80"E	35	31	35	88.57	0	11.43	0	0	0	0.43	0.29	diploid
26	Czech Rep.	Nový Stav	49°52'38.52"N	18°21'23.04"E	33	44	44	75.00	25.00	0.00	0	0	0	0.00	0.57	diploid

Table 2: continued

Nr.	Country	Population	Latitude	Longitude	n L	n R	N	% LR	% RR	% LL	%LLR	%LRR	%LLRR	HeL	HeR	classification
27	Czech Rep.	Zpupna Lhota	49°45'42.06"N	18°35'54.42"E	43	43	43	100.00	0.00	0.00	0	0	0	0.45	0.27	diploid
28	Czech Rep.	Albrechtičky	49°42'18.36"N	18°04'51.30"E	3*	16	16	18.75	81.25	0.00	0	0	0		0.59	diploid
29	Czech Rep.	Trnávka	49°40'54.90"N	18°11'5.22"E	54	44	58	68.97	6.90	24.14	0	0	0	0.41	0.17	diploid
30	Czech Rep.	Dobrá	49°40'36.96"N	18°23'30.18"E	55	24	55	43.64	0.00	56.36	0	0	0	0.47	0.18	diploid
31	Czech Rep.	Borovec	49°38'8.50"N	18°6'1.90"E	46	65	65	64.62	29.23	0.00	6	0	0	0.18	0.19	polyploid
32	Slovakia	Brodské	48°41'37.11"N	17°0'29.93"E		15	15	0	100.00	0	0	0	0		0.59	all-ridbundus
33	Slovakia	Šajdíkove Humence	48°39'14.30"N	17°17'1.19"E	29	32	32	37.50	9.38	0	53.13	0	0	0.34	0.35	polyploid
34	Slovakia	Kalaštov	48°37'55.26"N	17°15'12.30"E	35	25	35	91.43	0	8.57	0	0	0	0.42	0.34	diploid
35	Slovakia	Šaštin-Stráže	48°37'54.61"N	17°8'40.38"E	62	70	86	53.49	27.91	18.60	0	0	0	0.47	0.46	diploid
36	Slovakia	Kozi chrbát	48°37'53.58"N	17°17'41.28"E	67	67	67	43.28	0	0	56.72	0	0	0.39	0.36	polyploid
37	Slovakia	Borský Mikuláš	48°37'45.60"N	17°11'17.34"E	39	24	39	61.54	0	38.46	0	0	0	0.46	0.34	diploid
38	Slovakia	Lakšárska	48°33'39.40"N	17°10'1.7"E	26	25	26	96.15	0	3.85	0	0	0	0.47	0.40	diploid
39	Slovakia	Šprinclov Majer	48°12'59.85"N	17°11'15.51"E		10	10	0	100.00	0	0	0	0		0.47	all-ridbundus
40	Switzerland	Hellberg	47°17'45.72"N	8°48'48.38"E	14		14	21.43	0	78.57	0	0	0	0.69		diploid
41	Hungary	Zempleńi-hegpég	48°20'13.08"N	2139'04.80"E	14	15	15	93.33	6.67	0	0	0	0	0.30	0.12	diploid
42	Hungary	Sátoraljaùjhely	48°20'10.40"N	21°39'11.90"E	7	6	7	85.71	0	14.29	0	0	0	0.38	0.20	diploid
43	Hungary	Szabolsveresmat	48°17'32.04"N	22°1'57.65"E	7	7	7	100.00	0	0	0	0	0	0.39	0.16	diploid
44	Hungary	Kisvárda	48°13'50.37"N	22°3'39.53"E	9	9	9	100.00	0	0	0	0	0	0.43	0.19	diploid
45	Hungary	Várkötzi-morotva	48°7'0.00"N	21°26'60.00"E	7	6	7	85.71	0	14.29	0	0	0	0.43	0.23	diploid
46	Hungary	Kapuvar	47°40'3.60"N	17°8'2.70"E		61	61	0	100.00	0	0	0	0		0.57	all-ridbundus
47	Hungary	Osli	47°37'52.90"N	17°4'48.20"E		21	21	0	100.00	0	0	0	0		0.58	all-ridbundus
48	Hungary	Lakitelek	48°25'13.10"N	21°36'19.00"E		13	13	0	100.00	0	0	0	0		0.57	all-ridbundus
49	Ukraine	Shatsk	51°29'17.20"N	23°55'53.40"E	15	19	19	73.68	21.05	0	0	5.26	0	0.43	0.27	polyploid
50	Ukraine	Baturin	51°20'19.39"N	32°52'43.54"E	13	25	25	40.00	48.00	0	4.00	8.00	0	0.37	0.54	polyploid
51	Ukraine	Zhovtneve	50°8'3.25"N	36°45'58.65"E	19	22	22	77.27	13.64	0	9.09	0	0	0.16	0.52	polyploid
52	Ukraine	Gaidary Iskov	49°37'23.70"N	36°17'14.89"E	71	81	81	69.14	12.35	0	1.23	17.28	0	0.28	0.52	polyploid
53	Ukraine	Poltava	49°36'2.52"N	34°32'29.69"E		6	6	0	100.00	0	0	0	0		0.59	all-ridbundus
54	Ukraine	Buchach	49°3'52.70"N	25°22'59.16"E	17	20	26	38.46	34.62	23.08	3.85	0	0	0.40	0.68	polyploid
55	Ukraine	Zyurupinsk	46°36'50.80"N	32°43'10.13"E	5	21	23	13.04	78.26	8.70	0	0	0	0.25	0.65	diploid
56	Ukraine	Vilkovo	45°23'58.16"N	29°35'42.18"E		6	6	0	100.00	0	0	0	0		0.77	all-ridbundus

Table 2: continued

Nr.	Country	Population	Latitude	Longitude	n L	n R	N	% LR	% RR	% LL	%LLR	%LRR	%LLRR	HeL	HeR	classification
57	Slowenia	Kicar	46°26'36.50"N	15°55'45.70"E	33	19	33	57.58	0	42.42	0	0	0	0.51	0.41	diploid
58	Slowenia	Prilipe	45°52'42.80"N	15°37'31.30"E		43	43	0	100.00	0	0	0	0		0.73	all-ridbundus
59	Croatia	Zagreb	45°50'9.30"N	16°4'9.30"E		39	39	0	100.00	0	0	0	0		0.72	all-ridbundus
60	Croatia	Kutina	45°31'2.30"N	16°56'43.30"E		9	9	0	100.00	0	0	0	0		0.62	all-ridbundus
61	Romania	Oradea	47°3'46.35"N	21°56'12.74"E		5	5	0	100.00	0	0	0	0		0.64	all-ridbundus
62	Romania	Reci	45°50'28.68"N	25°55'48.39"E	8		8	37.50	0	62.50	0	0	0	0.45		diploid
63	Romania	Ciopeia	45°33'11.16"N	22°58'17.04"E		24	24	0	100.00	0	0	0	0		0.64	all-ridbundus
64	Romania	Sarmizegetuza	45°30'4.68"N	22°46'6.60"E		23	23	0	100.00	0	0	0	0		0.64	all-ridbundus
65	Romania	Nucşoara	45°20'15.54"N	24°46'55.64"E	7	5	7	14.29	57.14	85.71	0	0	0	0.40	0.65	diploid
66	Romania	Argineşti	44°34'32.38"N	23°24'47.96"E	7		7	28.57	0	71.43	0	0	0	0.42		diploid
67	Romania	Sinoe	44°33'40"N	28°45'48"E		10	10	0	100.00	0	0	0	0		0.79	all-ridbundus
68	Romania	Hinova	44°32'23.00"N	22°46'38.00"E	5	5	5	100.00	0	0	0	0	0		0.29	diploid
69	Romania	Scăpău	44°27'39.24"N	22°43'29.39"E		5	5	0	100.00	0	0	0	0		0.78	all-ridbundus
70	Romania	Basarabi	44°10'48"N	28°24'36.3"E		9	9	0	100.00	0	0	0	0		0.80	all-ridbundus
71	Bulgaria	Durankulak	43°42'2.88"N	28°34'31.80"E		14	14	0	100.00	0	0	0	0		0.67	all-ridbundus
72	Bulgaria	Bolata Dere	43°23'37.68"N	28°27'58.68"E		7	7	0	100.00	0	0	0	0		0.69	all-ridbundus

Table 3: Results from general linear model analyses for the dependent variable H_{eL} (genetic diversity in the L genome) and H_{eR} (genetic diversity in the R genome). Significant results are printed in bold.

	HeL				HeR			
Independent variable	df	coeff.	F	P	df	coeff.	F	P
Latitude	1	-0.025	-6.03	**< 0.0001**	1	-0.017	-3.52	**0.001**
Longitude	1	-0.004	-1.85	0.071	1	0.005	2.29	**0.026**
% LL in population	1	0.002	3.65	**0.001**	-	-	-	-
% RR in population	-	-	-	-	1	0.003	9.24	**< 0.0001**
% polyploid hybrids in population	1	-0.001	-1.13	0.264	1	0.003	4.32	**< 0.0001**
Error	46				62			

Table 4: Pairwise F_{ST} values for all populations containing polyploid hybrids. Populations are sorted from left to right/top to bottom with increasing geographic longitude. The upper diagonal (green colors) denotes values for the R genome, the lower diagonal (brownish color) for the L genome. Light shades indicate little and moderate differentiation, darker shades indicate great differentiation. According to Wright (1978): 0≤ F_{ST} <0.05 mean little genetic differentiation, 0.05≤ F_{ST} <0.15 moderate, 0.15≤ F_{ST} <0.25 great, 0.25≤ F_{ST} very great genetic differentiation.

	Untermassfeld	KluetzerWinkel	Fehmarn	Altenhausen	Schönermark	Grammentin	Teschendorf	Genarp	Ruegen	Usedom	Cottbus	Doebern	Kolczewo	Wysoka	Bornholm	Sanie	SajdikoveHum.	KoziChrbat	Borovec	Shatsk	Buchach	Baturin	Gaidary Iskov	Zhovtneve
Untermassfeld		0.23	0.33	0.11	0.27	0.26	0.35	0.47	0.31	0.17	0.16	0.22	0.14	0.19	0.23	0.23	0.20	0.17	0.34	0.26	0.22	0.27	0.40	0.45
KluetzerWinkel	0.27		0.29	0.18	0.33	0.35	0.40	0.61	0.30	0.26	0.25	0.26	0.21	0.19	0.29	0.26	0.25	0.23	0.31	0.34	0.32	0.34	0.42	0.50
Fehmarn	0.36	0.34		0.25	0.35	0.48	0.44	0.70	0.37	0.37	0.32	0.37	0.44	0.35	0.31	0.36	0.33	0.35	0.38	0.41	0.36	0.42	0.47	0.57
Altenhausen	0.19	0.16	0.26		0.22	0.23	0.30	0.50	0.27	0.14	0.10	0.20	0.15	0.14	0.19	0.22	0.19	0.19	0.32	0.25	0.18	0.25	0.37	0.42
Schönermark	0.24	0.10	0.28	0.08		0.18	0.20	0.27	0.33	0.18	0.19	0.16	0.24	0.23	0.29	0.25	0.33	0.32	0.37	0.33	0.29	0.30	0.41	0.48
Grammentin	0.22	0.10	0.38	0.13	0.08		0.20	0.40	0.46	0.25	0.17	0.26	0.29	0.25	0.36	0.31	0.37	0.35	0.44	0.43	0.34	0.38	0.46	0.54
Teschendorf	0.20	0.09	0.25	0.09	0.12	0.07		0.42	0.44	0.30	0.28	0.34	0.40	0.34	0.42	0.37	0.41	0.42	0.50	0.41	0.29	0.38	0.42	0.51
Genarp	0.16	0.44	0.52	0.26	0.29	0.38	0.33		0.74	0.58	0.31	0.38	0.64	0.54	0.57	0.43	0.61	0.53	0.62	0.66	0.56	0.60	0.59	0.76
Ruegen	0.23	0.18	0.28	0.13	0.10	0.11	0.12	0.37		0.28	0.33	0.36	0.33	0.28	0.26	0.39	0.31	0.32	0.37	0.38	0.30	0.32	0.41	0.53
Usedom	0.22	0.22	0.30	0.13	0.14	0.16	0.11	0.35	0.09		0.19	0.21	0.11	0.12	0.18	0.29	0.26	0.25	0.36	0.21	0.16	0.17	0.29	0.38
Cottbus	0.18	0.50	0.60	0.36	0.35	0.45	0.38	0.13	0.44	0.42		0.21	0.17	0.20	0.21	0.20	0.26	0.24	0.36	0.32	0.21	0.28	0.41	0.47
Doebern	0.23	0.34	0.45	0.28	0.27	0.31	0.29	0.28	0.28	0.28	0.28		0.15	0.22	0.29	0.17	0.25	0.21	0.26	0.28	0.38	0.40	0.48	0.59
Kolczewo	0.23	0.43	0.58	0.27	0.30	0.42	0.31	0.39	0.39	0.34	0.51	0.44		0.10	0.18	0.20	0.22	0.16	0.24	0.26	0.20	0.25	0.38	0.47
Wysoka	0.18	0.31	0.43	0.18	0.19	0.21	0.23	0.23	0.20	0.16	0.26	0.20	0.29		0.20	0.24	0.22	0.22	0.30	0.26	0.22	0.25	0.35	0.42
Bornholm	0.39	0.59	0.67	0.41	0.46	0.58	0.46	0.58	0.56	0.50	0.62	0.58	0.60	0.49		0.29	0.25	0.25	0.34	0.28	0.23	0.24	0.37	0.46
Sanie	0.10	0.26	0.35	0.20	0.23	0.21	0.24	0.28	0.19	0.17	0.30	0.26	0.27	0.16	0.38		0.24	0.20	0.27	0.31	0.32	0.35	0.45	0.55
SajdikoveHum.	0.39	0.47	0.47	0.45	0.43	0.49	0.43	0.62	0.42	0.35	0.64	0.54	0.59	0.50	0.62	0.27		0.03	0.21	0.19	0.29	0.31	0.42	0.50
KoziChrbat	0.38	0.44	0.42	0.42	0.42	0.44	0.41	0.55	0.37	0.33	0.57	0.49	0.53	0.45	0.54	0.25	0.04		0.24	0.21	0.31	0.30	0.43	0.52
Borovec	0.07	0.38	0.44	0.25	0.27	0.32	0.28	0.07	0.29	0.26	0.07	0.21	0.29	0.15	0.46	0.18	0.50	0.46		0.34	0.42	0.45	0.50	0.62
Shatsk	0.11	0.25	0.38	0.18	0.18	0.20	0.22	0.37	0.20	0.16	0.44	0.32	0.31	0.19	0.50	0.01	0.25	0.23	0.24		0.22	0.28	0.37	0.48
Buchach	0.19	0.24	0.35	0.22	0.20	0.22	0.22	0.41	0.18	0.16	0.45	0.31	0.33	0.24	0.47	0.13	0.30	0.28	0.29	0.12		0.15	0.23	0.29
Baturin	0.30	0.32	0.53	0.32	0.26	0.37	0.37	0.59	0.38	0.40	0.64	0.49	0.52	0.45	0.64	0.22	0.45	0.41	0.48	0.16	0.28		0.16	0.23
Gaidary Iskov	0.48	0.54	0.74	0.59	0.56	0.65	0.59	0.74	0.62	0.57	0.78	0.65	0.82	0.61	0.77	0.37	0.56	0.49	0.62	0.45	0.45	0.59		0.10
Zhovtneve	0.49	0.50	0.69	0.56	0.52	0.60	0.56	0.70	0.59	0.54	0.76	0.65	0.73	0.61	0.75	0.37	0.54	0.47	0.62	0.42	0.43	0.51	0.40	

Table 5: Haplotypes, sample sizes and genotype distribution of main clusters (compare Figure 8) defined by phylogenetic analysis of mtDNA. Genotypes have been inferred from microsatellite analysis based on 18 neutral markers.

Cluster	Haplotype	No. Localities (samples)	Countries	Parental genotypes	Hybrid genotypes
	L1	47 (369)	D;S;DK;PL;LT;LV;EST;CZ;SK;RO;BG;UA	RR, LL	LLR, LR, LRR
	L2	9 (34)	D; S		LLR, LR, LRR
	L3	2 (19)	D	RR	LLR, LR, LRR
	L5	1 (11)	D		LR
	L6	12 (36)	D;PL;CZ;SK;SLO;H;UA	RR, LL	LLR, LR
	L9	1 (3)	D		LR
	L10	2 (5)	D	RR	LR
	L11	1 (7)	D		LLR, LR, LRR
	L12	1 (10)	D		LLR, LR, LRR
	L13	4(4)	D;PL;DK;EST	RR	LR
	L15	6(30)	D;DK;PL;LV;RO	RR, LL	LLR, LR
	L16	3(4)	PL;CZ	RR, LL	
les-1	L18	1(3)	CZ	LL	
	L21	2(11)	SK		LLR , LR
	L22	1(2)	SK		LLR
	L24	1(2)	ROM	LL	
	L25	2(5)	S;ROM	LL	LR
	L27	1(11)	EST	LL	
	L28	1(1)	LV		LR
	L29	1(11)	DK	LL	LR
	L30	1(1)	D	LL	
	L31	1(1)	D	LL	
	L32	3(4)	D;LV		LLR, LR
	L33	1(2)	EST	LL	
	L35	1(1)	PL		LR
	L26	1(4)	PL		LLR, LR
	L40	1(1)	PL		LR
	L36	1(3)	LT	LL	LR
	L37	2(11)	LT;LV	LL	LR
	L38	1(2)	LV	LL	
	L39	2(20)	D;DK		LLR, LR, LRR
	L4	9(30)	D;PL;CZ;SLO;LT	LL	LLR, LR, LRR
	L7	3(7)	D;H;ROM	LL	LR
	L8	1(1)	D		LRR
	L14	24(125)	PL;CZ;SK;H;SLO;ROM;UA;LT	LL	LLR, LR
les-2	L17	1(4)	CZ	LL	
	L19	2(5)	CZ	LL	LR
	L20	2(3)	DK, CZ		LR
	L23	2(2)	SK	LL	LLR
	L34	1(2)	LT	LL	LR
bergeri-type	B1	3 (14)	D, CH	LL	LR, LRR*
	B2	1(1)	CH	LL	
begriagae-type	A1	1(1)	UA		LR
	R1	48(166)	D;PL;CZ;SK;H;ROM;BG;UA;LV	RR	LR
	R5	1(3)	SK	RR	
	R7	2(25)	HR	RR	
	R12	1(1)	H	RR	
rid-1	R13	2(3)	H	RR	
	R14	1(1)	H	RR	
	R15	1(3)	H	RR	
	R25	2(2)	UA		LR
	R26	2(8)	BG	RR	
	R28	1(1)	BG	RR	
	R2	1(2)	CZ		LR
	R4	6(11)	SK;H;ROM;BG	RR	LR
	R9	1(2)	HR	RR	
rid-2	R10	1(1)	HR	RR	
	R16	1(2)	H	RR	
	R20	1(1)	ROM	RR	
	R27	3(3)	BG	RR	

Table 5: continued

Cluster	Haplotype	No. Localities (samples)	Countries	Parental genotypes	Hybrid genotypes
	R3	1(2)	CZ		LR
	R6	28(82)	D;SLO;ROM;BG;UA;LT;LV	RR	LR, LRR**
	R8	1(3)	HR	RR	
	R11	1(1)	HR	RR	
	R17	3(3)	ROM;UA	RR	LR
	R18	1(3)	ROM	RR	
rid-2	R19	1(1)	ROM	RR	
	R21	1(2)	ROM	RR	
	R22	1(2)	ROM	RR	
	R23	4(5)	UA	RR	LR
	R24	3(8)	UA	RR	LR
	R29	1(2)	UA	RR	
	R30	1(2)	LT	RR	
	R32	1(1)	LT	RR	
	R31	1(2)	LT	RR	

* 1 individual in Untermassfeld, Germany
** 5 individuals in Gaidary, Ukraine

Figures

Figure 1: Sample locations for microsatellite analyses. Red circles indicate populations where polyploid hybrids were found, white circles indicate populations where diploid hybrids occurred, green circles denote pure *P. ridibundus* populations, pink circles indicate pure *P. lessonae* populations.

Figure 2: H_{eL} (genetic diversity in the L genome) plotted against geographic latitude (a) and longitude (b) for different types of populations.

Figure 3: H_{eR} (genetic diversity in the R genome) plotted against geographic latitude (a) and longitude (b) for different types of populations.

Figure 4: Isolation by distance in the L (a) and R genome (b). Data points indicate pairwise genetic distances between populations (given as Nei's Ds based on microsatellite analysis). Populations were pooled irrespective of genotype composition.

Figure 5: Single-linkage clustering analysis based on Euclidean distances of Nei's D (L genome) between all polyploid populations. Population numbers are given on the right and correspond to numbers in Table 2. Brackets on the right indicate association to a cluster.

Figure 6: Single-linkage clustering analysis based on Euclidean distances of Nei's D (R genome) between all polyploid populations. Population numbers are given on the right and correspond to numbers in Table 2. Brackets on the right indicate association to a cluster.

Figure 7: Genetic structuring of polyploid populations resulting from cluster analyses based on pairwise genetic distances (Nei's D) of microsatellite genotypes for the L genome (a) and R genome (b). Same colors indicate association within a cluster. The black colors in the top map (a) correspond to populations that could not be clearly assigned to a cluster (compare Figure 5).

Figure 8: Localities of samples for mtDNA analysis for *lessonae* (a) and *ridibundus* (b) types. Symbol colors refer to different population types where the respective mtDNA-type was found (yellow = polyploid populations, mtDNA-type found in polyploid and diploid hybrids and parental species, white = diploid populations, mtDNA-type found only in diploid hybrids and parental species, pink = polyploid populations, mtDNA type found only in diploid hybrids and parental species, blue = all-ridibundus population, green = all-lessonae population).

Figure 9: Phylogenetic relationships among 75 mtDNA haplotypes, as inferred from Maximum Likelihood Analysis. The units on the scale bar are expected mutations per site. Filled symbols indicate haplotypes found in polyploids. Values higher than 95 are considered statistically significant. Bootstrap values below 80 are not shown.

Figure 1

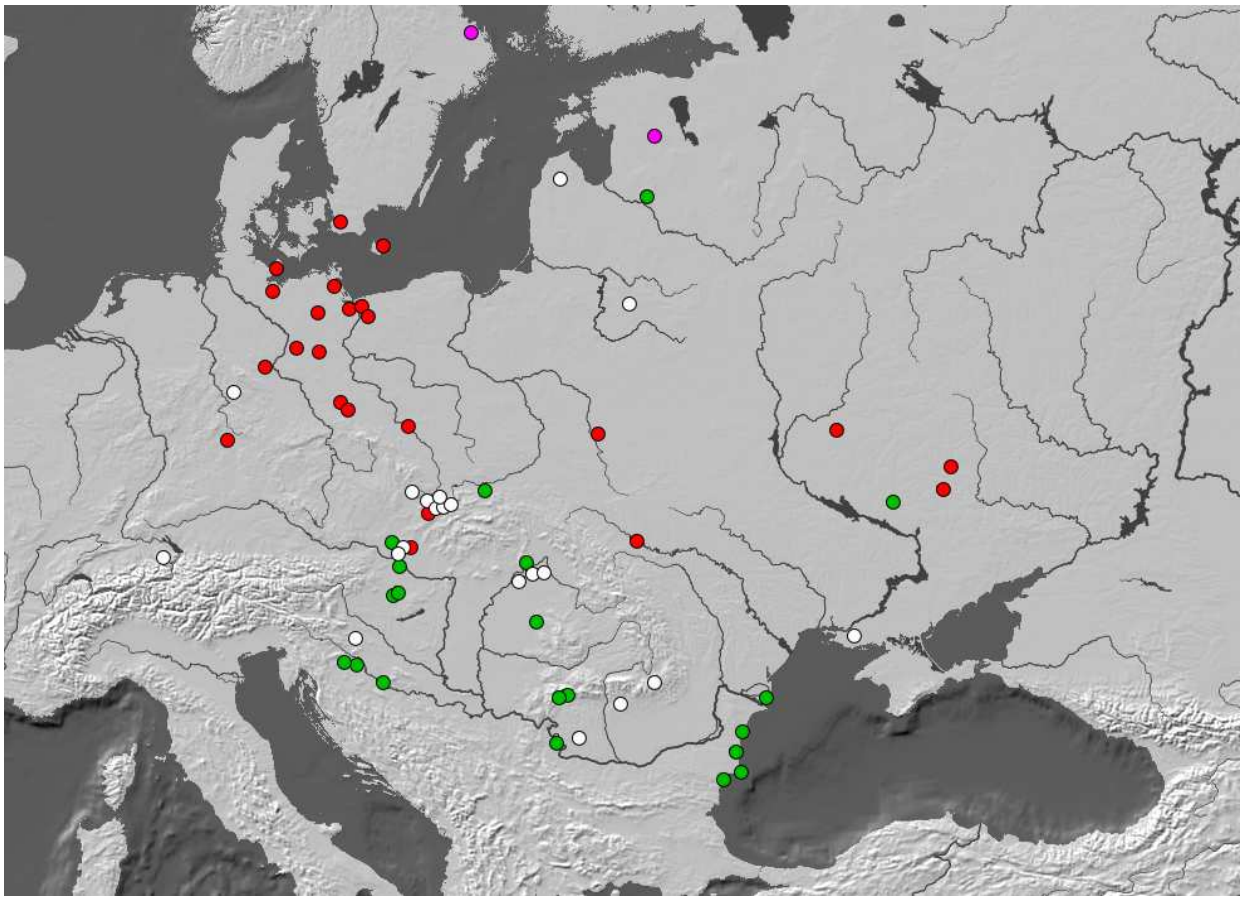


Figure 2

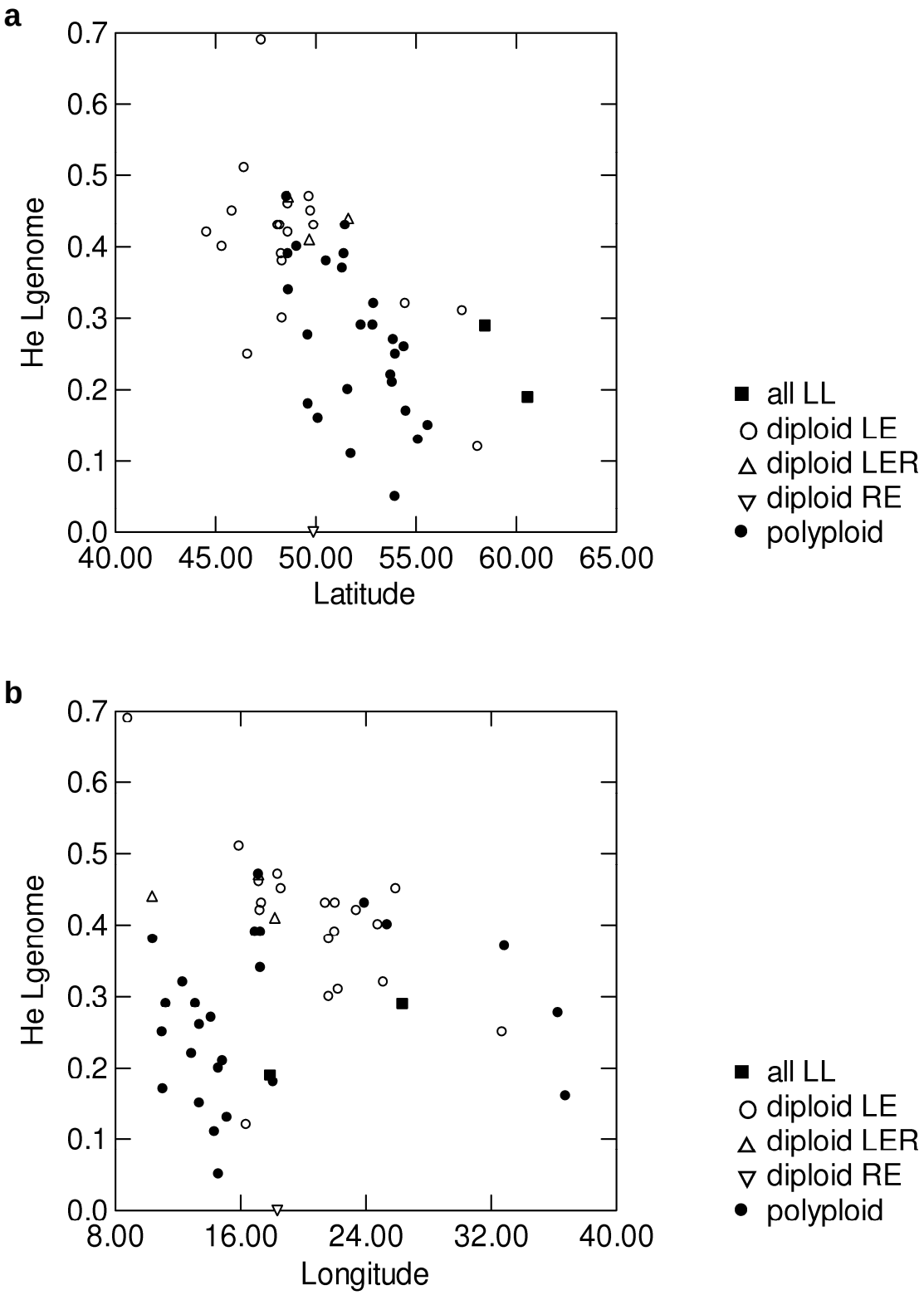
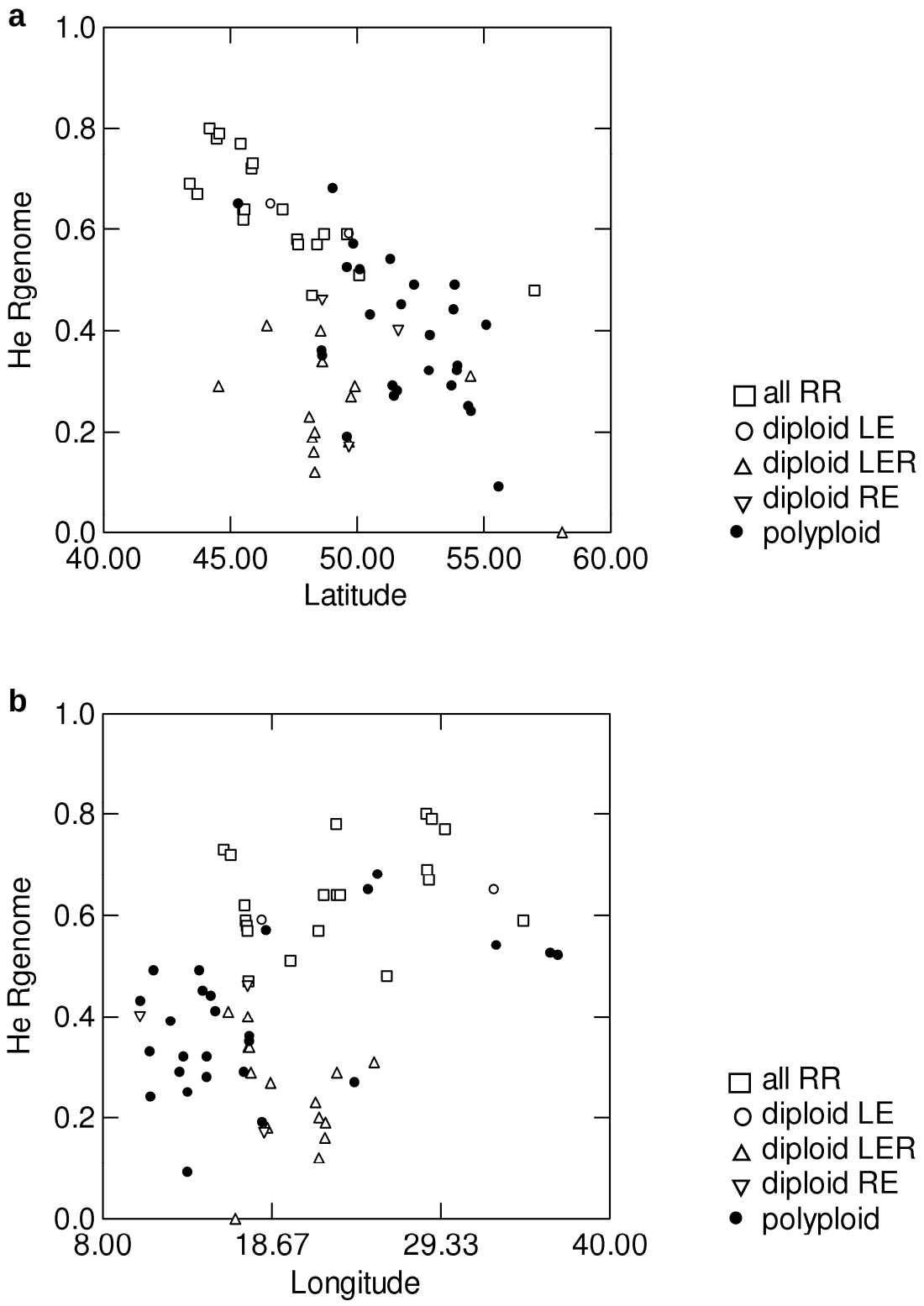

a
He Lgenome
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
40.00
45.00
50.00
55.00
60.00
65.00
Latitude
all LL
diploid LE
diploid LER
diploid RE
polyploid
b
He Lgenome
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
8.00
16.00
24.00
32.00
40.00
Longitude
all LL
diploid LE
diploid LER
diploid RE
polyploid

Figure 3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0.6
0.4
0.2
0.0
He Rgenome
40.00
45.00
50.00
55.00
60.00
Latitude
all RR
diploid LE
diploid LER
diploid RE
polyploid
b
1.0
0.8
0.6
0.4
0.2
0.0
He Rgenome
8.00
18.67
29.33
40.00
Longitude
all RR
diploid LE
diploid LER
diploid RE
polyploid

Figure 4

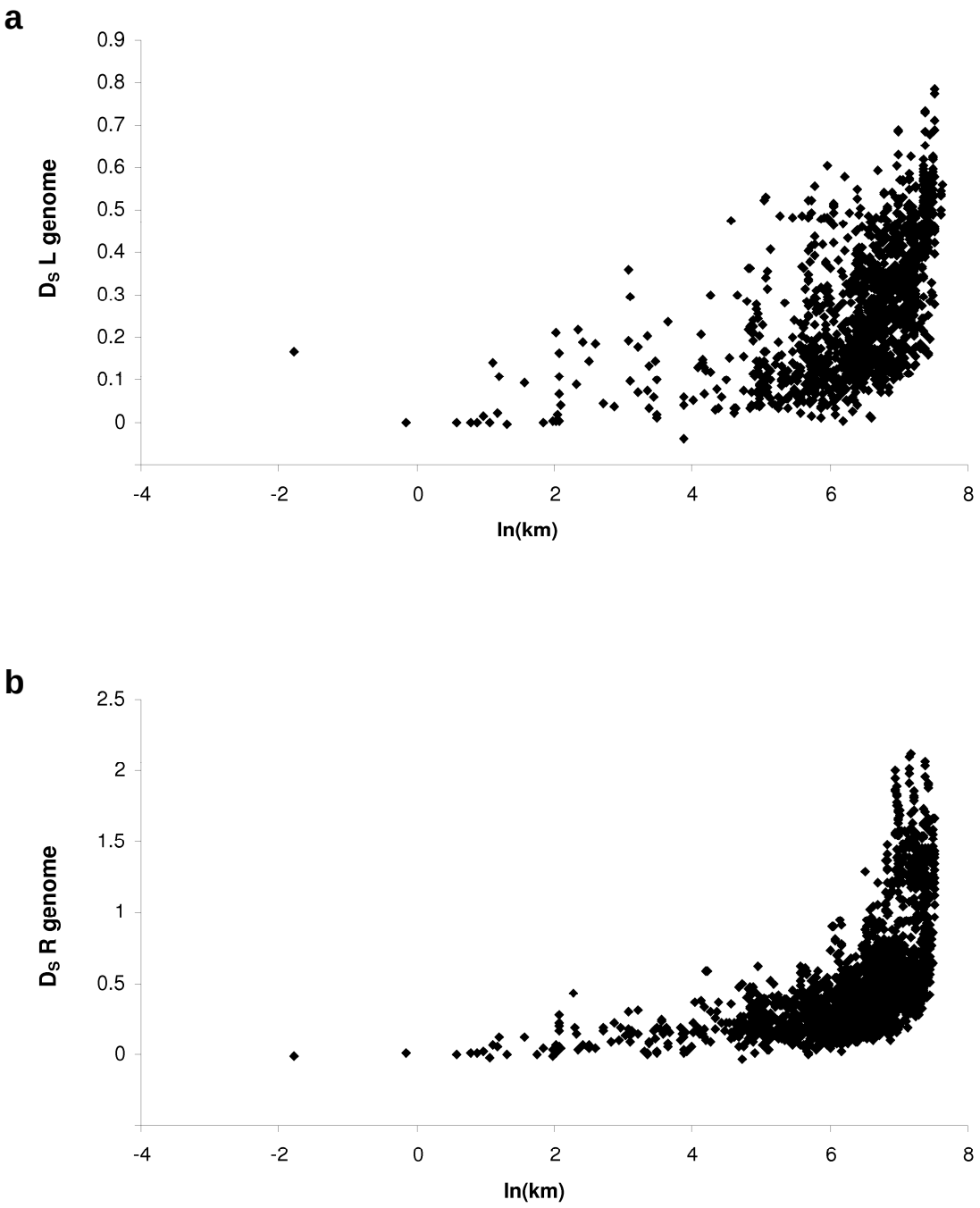

a
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
D_S L genome
-4
-2
0
2
4
6
8
ln(km)
b
2.5
2
1.5
1
0.5
0
D_S R genome
-4
-2
0
2
4
6
8
ln(km)

Figure 5

L genome

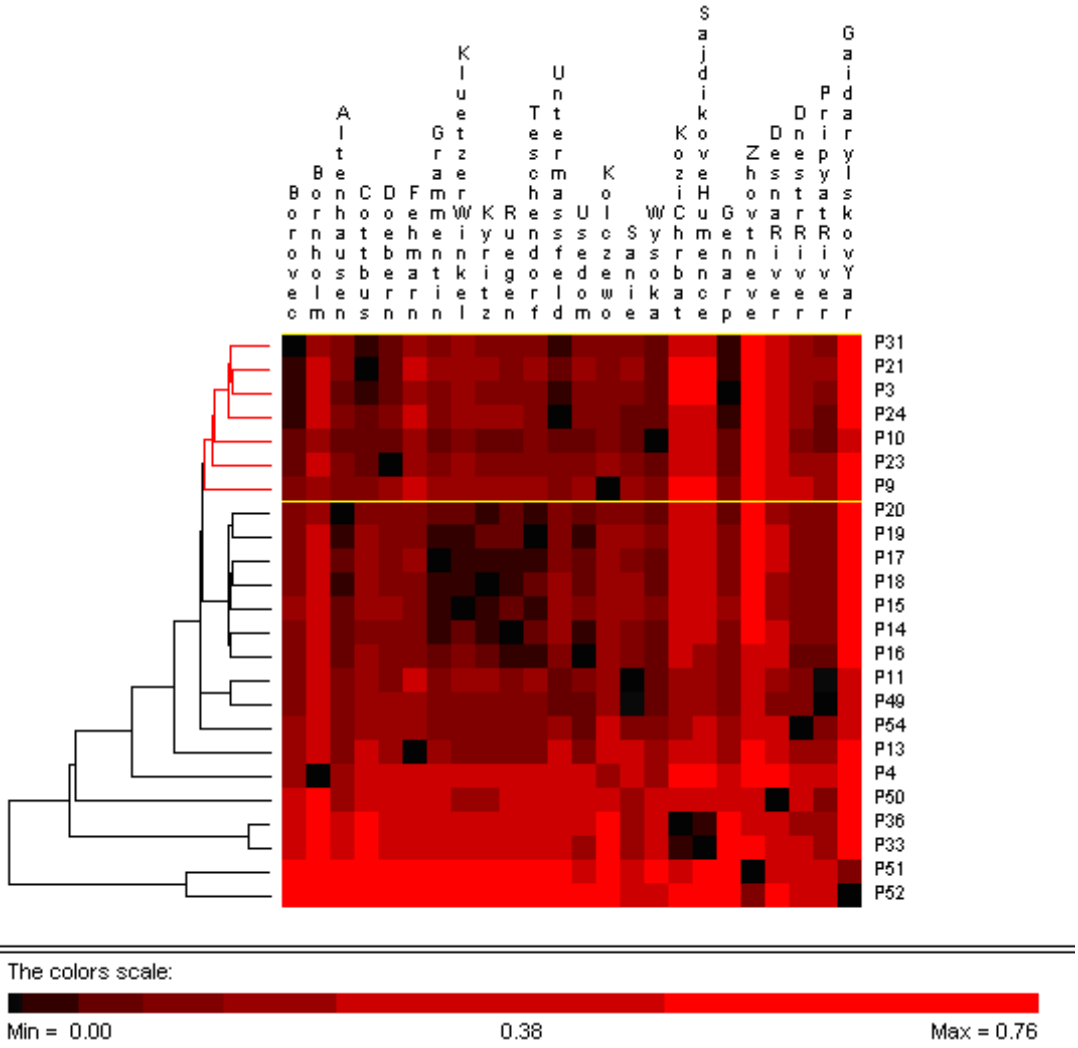


Figure 6

R genome

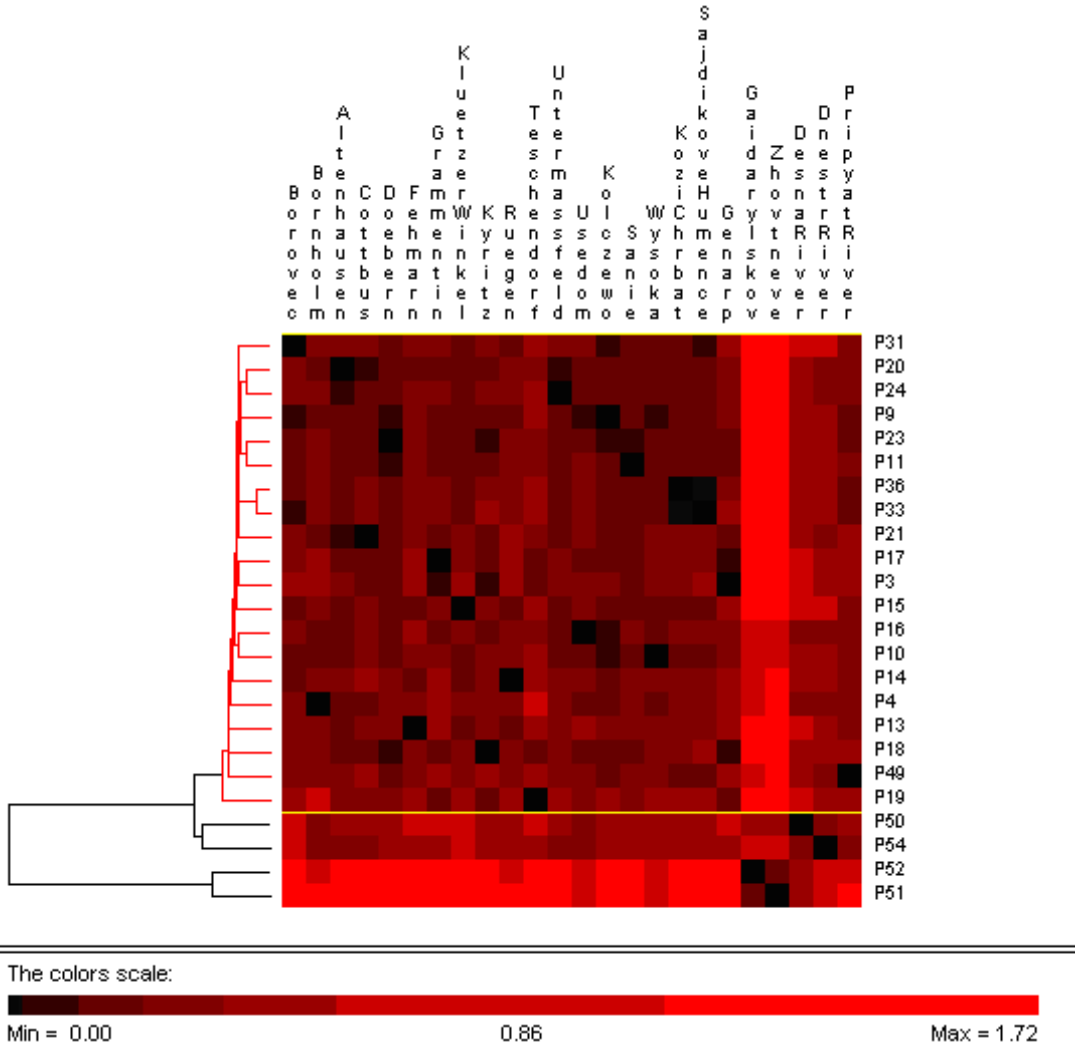
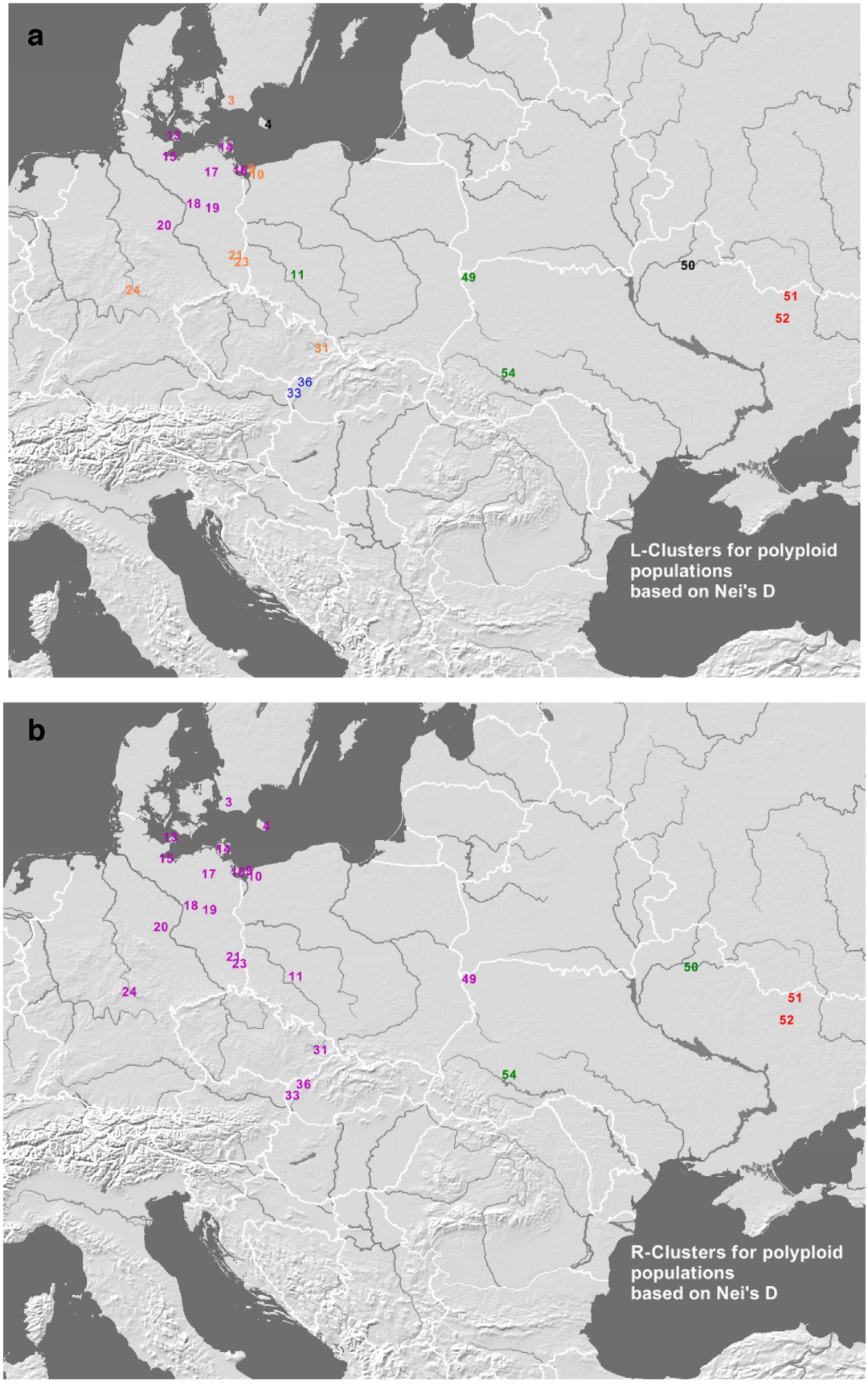


Figure 7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3
4
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14
15
17
18
19
20
21
23
11
24
49
50
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52
31
54
36
33
L-Clusters for polyploid
populations
based on Nei's D
b
3
4
13
14
15
17
18
19
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51
52
31
54
36
33
R-Clusters for polyploid
populations
based on Nei's D

Figure 8

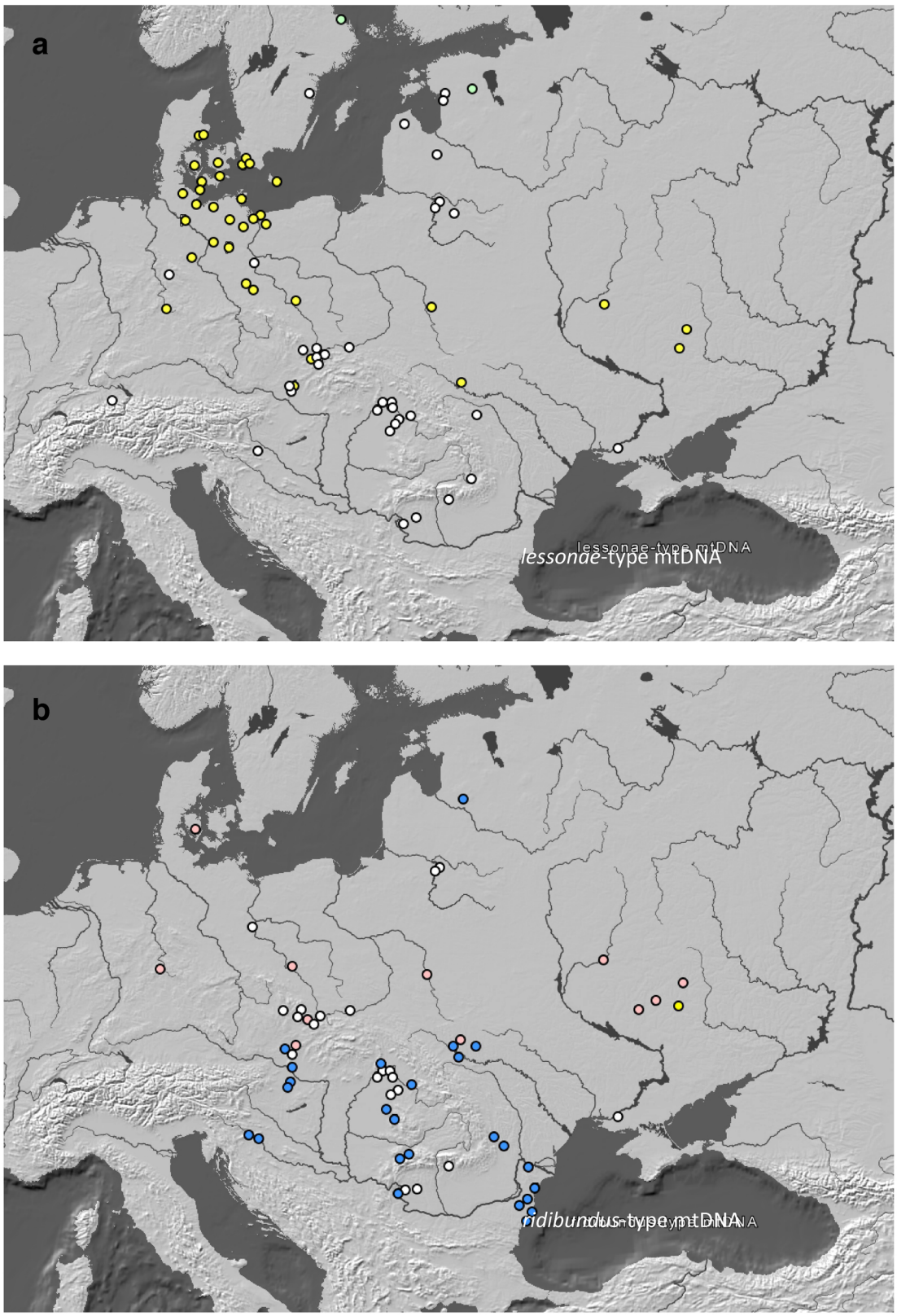
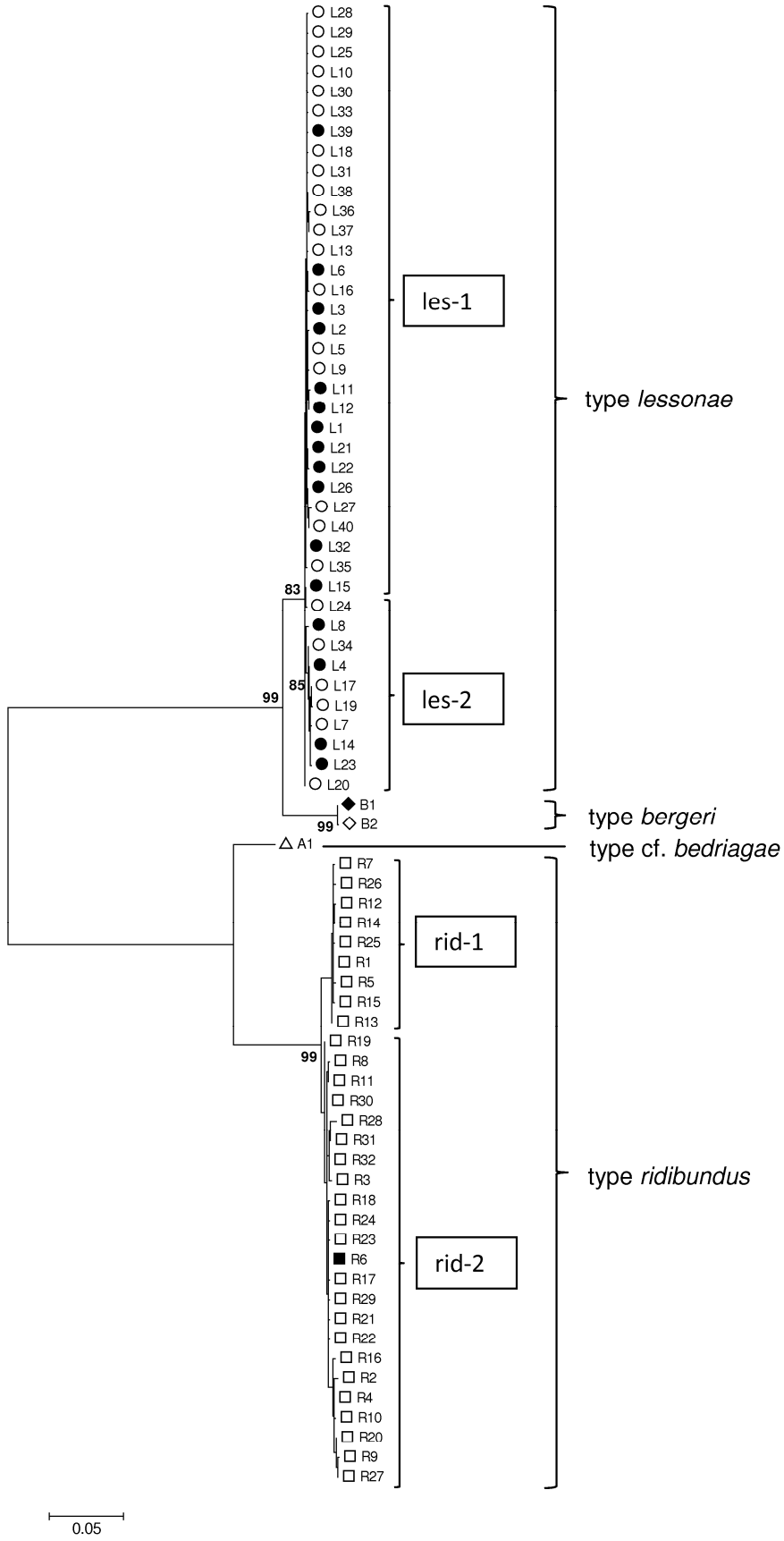


Figure 9


L28
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L6
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L2
L5
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L4
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B1
B2
A1
R7
R26
R12
R14
R25
R1
R5
R15
R13
R19
R8
R11
R30
R28
R31
R32
R3
R18
R24
R23
R6
R17
R29
R21
R22
R16
R2
R4
R10
R20
R9
R27
83
85
99
99
99
les-1
les-2
rid-1
rid-2
type lessonae
type bergeri
type cf. bedriagae
type ridibundus
0.05

Appendix:

A1: Populations, geographic coordinates and sample sizes of samples used for mtDNA analysis. The column "Microsat.Nr*" refers to the numbering of populations in Table 2, where only populations used in microsatellite analyses are listed. Types of mtDNA are abbreviated as follows: les = *lessonae*-type, rid = *ridibundus*-type, ber = *berger*i-type, cf. bed = cf. *bedriagae*-type.

Nr.	Microsat.Nr.* (Table 2)	Country	Population	Latitude	Longitude	classification	N	n type mitochondrial DNA		
								les	rid	berg	cf. bed
SE-1	1	Sweden	Uppsala	60°33'24.52"N	17°51'46.04"E	all-lessonae	13		13		
SE-2	2	Sweden	Östergötland	58°5'38.59"N	16°22'28.68"E	diploid	11		11		
SE-3	3	Sweden	Genarp	55°36'34.00"N	13°23'19.00"E	polyploid	16		16		
SE-4		Sweden	Skane 001	55°35'17.80"N	13°21'07.67"E	polyploid	3		3		
SE-5		Sweden	Skane 032	55°34'03.00"N	13°12'53.00"E	polyploid	4		4		
SE-6		Sweden	Skane 050	55°29'33.00"N	13°08'02.00"E	polyploid	5		5		
SE-7		Sweden	Skane 159	55°23'17.04"N	13°26'50.99"E	polyploid	5		5		
DK-1	4	Denmark	Jutland	56°09'02.99"N	10°31'57.00"E	polyploid	33		33		
DK-2		Denmark	Bornholm	55°07'28.72"N	15°08'58.57"E	polyploid	31		31		
DK-3		Denmark	Sjelland	55°27'47.71"N	11°43'17.39"E	polyploid	4		4		
DK-4		Denmark	Funen	55°07'09.00"N	10°30'30.00"E	polyploid	15		15		
DK-5		Denmark	Saeland	55°00'30.99"N	12°00'16.99"E	polyploid	28		28		
DK-6		Denmark	Lolland	54°47'30.00"N	11°00'11.00"E	polyploid	16		16		
EE-1	5	Estonia	Laeva	58°25'41.99"N	26°19'7.99"E	all-lessonae	12		12		
EE-2		Estonia	Pärnu	58°23'05.00"N	24°31'07.00"E	diploid	1		1		
EE-3		Estonia	Hara	58°05'16.01"N	24°29'39.00"E	diploid	11		11		
LV-1	6	Latvia	Stickli	57°19'41.00"N	22°15'21.99"E	diploid	8		8		
LV-2	7	Latvia	Jurmala	56°59'36.99"N	25°55'22.00"E	all-ridbundus	6	6			
LT1	8	Lithuania	LT-B	56°22'52.00"N	24°10'06.00"E	diploid	4		4		
LT-2		Lithuania	LT-K	54°47'49.00"N	24°15'05.00"E	diploid	13	7	6		
LT-3		Lithuania	Dasunikeskes	54°42'49.00"N	24°05'54.00"E	diploid	2	1	1		
LT-4		Lithuania	Baltoji Voke	54°28'44.00"N	25°7'59.00"E	diploid	23		23		
PL-1	9	Poland	Kolczewo	53°57'54.0"N	14°36'34.10"E	polyploid	8		8		
PL-2	10	Poland	Wysoka Baczyslaw	53°49'53.47"N	14°51'51.53"E	polyploid	25		25		
PL-3	11	Poland	Sanie	51°25'38.10"N	16°56'57.84"E	polyploid	49	4	45		
PL-4	12	Poland	Krakow	50°5'3.60"N	19°50'26.46"E	all-ridbundus	11	1	10		
D-1	13	Germany	Fehmarn	54°31'9.984"N	11°3'13.96"E	polyploid	13		13		
D-2	14	Germany	Rügen	54°25'1.44"N	13°23'45.39"E	polyploid	16		16		
D-3		Germany	Preetz	54°14'50.30"N	10°11'26.63"E	polyploid	2		2		

A1: continued

Nr.	Microsat.Nr.* (Table 2)	Country	Population	Latitude	Longitude	classification	N	n type mitochondrial DNA les	rid	berg	cf. bed
D-4		Germany	Hanstorf	54°02'26.96"N	11°54'40.20"E	polyploid	2		2		
D-5	15	Germany	Klützer Winkel	53°59'23.34"N	11°0'37.02"E	polyploid	14		14		
D-6	16	Germany	Usedom	53°52'44.82"N	14°8'20.58"E	polyploid	8		8		
D-7	17	Germany	Grammentin	53°45'26.72"N	12°53'41.04"E	polyploid	5		5		
D-8		Germany	Rothemühl	53°34'26.00"N	13°46'04.00"E	polyploid	2		2		
D-9		Germany	Gülzow	53°26'41.14"N	10°30'04.72"E	polyploid	6		6		
D-10	18	Germany	Schönermark	52°54'7.08"N	12°19'15.50"E	polyploid	2		2		
D-11		Germany	Doerverdem	52°53'47.00"N	9°16'26.00"E	polyploid	15		15		
D-12	19	Germany	Teschendorf	52°51'53.03"N	13°8'40.38"E	polyploid	10		10		
D-13		Germany	Lebus	52°24'50.76"N	14°32'31.92"E	diploid	7	2	5		
D-14	20	Germany	Altenhausen	52°16'40.0"N	11°15'15.01"E	polyploid	9		9		
D-15	21	Germany	Cottbus	51°46'24.30"N	14°21'19.14"E	polyploid	43		43		
D-16	22	Germany	Herzberg	51°37'36.66"N	10°21'15.06"E	diploid	7		2	5	
D-17	23	Germany	Döbern	51°36'38.22"N	14°36'15.60"E	polyploid	46		46		
D-18	24	Germany	Untermassfeld	50°32'21.06"N	10°24'28.44"E	polyploid	39	2	32	5	
CZ-1	25	Czech Rep.	Břidličná	49°55'00.40"N	17°21'39.80"E	diploid	5		5		
CZ-2	26	Czech Rep.	Nový Stav	49°52'38.52"N	18°21'23.04"E	diploid	24	19	5		
CZ-3	27	Czech Rep.	Zpupna Lhota	49°45'42.06"N	18°35'54.42"E	diploid	22		22		
CZ-4	28	Czech Rep.	Albrechtičky	49°42'18.36"N	18°04'51.30"E	diploid	5	5			
CZ-5	29	Czech Rep.	Trnávka	49°40'54.90"N	18°11'5.22"E	diploid	19	2	17		
CZ-6	30	Czech Rep.	Dobrá	49°40'36.96"N	18°23'30.18"E	diploid	39		39		
CZ-7	31	Czech Rep.	Borovec	49°38'8.50"N	18°6'1.90"E	polyploid	30	4	26		
SK-1	32	Slovakia	Brodské	48°41'37.11"N	17°0'29.93"E	all-ridbundus	5	5			
SK-2	33	Slovakia	Šajd. Humence	48°39'14.30"N	17°17'1.19"E	polyploid	12	1	11		
SK-3	34	Slovakia	Kalaštov	48°37'55.26"N	17°15'12.30"E	diploid	10		10		
SK-4	35	Slovakia	Šaštin-Stráže	48°37'54.61"N	17°8'40.38"E	diploid	3		3		
SK-5	36	Slovakia	Kozi chrbát	48°37'53.58"N	17°17'41.28"E	polyploid	10		10		
SK-6	38	Slovakia	Lakšárska	48°33'39.40"N	17°10'1.7"E	diploid	4		4		
SK-7	39	Slovakia	Šprinclov Majer	48°12'59.85"N	17°11'15.51"E	all-ridbundus	9	9			
CH-1	40	Switzerland	Hellberg	47°17'45.72"N	8°48'48.38"E	diploid	5		2	3	

A1: continued

Nr.	Microsat.Nr.* (Table 2)	Country	Population	Latitude	Longitude	classification	N	n type mitochondrial DNA			
								les	rid	berg	cf. bed
HUN-1	41	Hungary	Zempleńi-hegpég	48°20'13.08"N	2139'04.80"E	diploid	15	2	13		
HUN-2	42	Hungary	Sátoraljaùjhely	48°20'10.40"N	21°39'11.90"E	diploid	7	2	5		
HUN-3	43	Hungary	Szabolsveresmat	48°17'32.04"N	22°1'57.65"E	diploid	6	1	5		
HUN-4	44	Hungary	Kisvárda	48°13'50.37"N	22°3'39.53"E	diploid	9	5	4		
HUN-5	45	Hungary	Várkötzi-morotva	48°7'0.00"N	21°26'60.00"E	diploid	7	2	5		
HUN-6	46	Hungary	Kapuvar	47°40'3.60"N	17°8'2.70"E	all-ridbundus	27	27			
HUN-7	47	Hungary	Osli	47°37'52.90"N	17°4'48.20"E	all-ridbundus	12	12			
HUN-8	48	Hungary	Lakitelek	48°25'13.10"N	21°36'19.00"E	all-ridbundus	12	12			
UA-1	49	Ukraine	Shatsk	51°29'17.20"N	23°55'53.40"E	polyploid	16	9	7		
UA-2	50	Ukraine	Baturin	51°20'19.39"N	32°52'43.54"E	polyploid	8	6	2		
UA-3	51	Ukraine	Zhovtneve	50°8'3.25"N	36°45'58.65"E	polyploid	9	9			
UA-4	52	Ukraine	Gaidary Iskov Yar	49°37'23.70"N	36°17'14.89"E	polyploid	46	45			1
UA-5	53	Ukraine	Poltava	49°36'2.52"N	34°32'29.69"E	all-ridbundus	4	4			
UA-6	54	Ukraine	Buchach	49°3'52.70"N	25°22'59.16"E	polyploid	4	4			
UA-7	55	Ukraine	Zyurupinsk	46°36'50.80"N	32°43'10.13"E	diploid	2	2			
UA-8	56	Ukraine	Vilkovo	45°23'58.16"N	29°35'42.18"E	all-ridbundus	6	6			
SL-1	57	Slowenia	Kicar	46°26'36.50"N	15°55'45.70"E	diploid	12		12		
SL-2	58	Slowenia	Prilipe	45°52'42.80"N	15°37'31.30"E	all-ridbundus	9	9			
HR-1	59	Croatia	Zagreb	45°50'9.30"N	16°4'9.30"E	all-ridbundus	32	32			
ROM-1		Romania	Dersca	47°59'21.71"N	26°12'48.10"E	diploid	2		2		
ROM-2		Romania	Livada	47°51'54.63"N	23°07'10.47"E	diploid	3	1	2		
ROM-3		Romania	Foieni	47°41'53.70"N	22°23'14.07"E	diploid	4	3	1		
ROM-4		Romania	Resighea	47°35'49.66"N	22°17'47.35"E	diploid	4		4		
ROM-5		Romania	Şimian	47°29'21.00"N	22°05'17.20"E	diploid	5	3	2		
ROM-6	61	Romania	Oradea	47°03'46.35"N	21°56'12.74"E	all-ridbundus	4	4			
ROM-7		Romania	Câmpani de Pomezeu	46°48'02.08"N	22°19'00.22"E	ridibundus	4	4			
ROM-8		Romania	Căiuţi	46°11'00.10"N	26°55'42.95"E	ridibundus	4	4			
ROM-9		Romania	Tecuci	45°50'48.03"N	27°26'05.82"E	ridibundus	3	3			
ROM-10	62	Romania	Reci	45°50'28.68"N	25°55'48.39"E	diploid	7		7		
ROM-11	63	Romania	Ciopeia	45°33'11.16"N	22°58'17.04"E	all-ridbundus	9	9			
ROM-12	64	Romania	Sarmizegetuza	45°30'04.68"N	22°46'06.60"E	all-ridbundus	10	10			

A1: continued

Nr.	Microsat.Nr.* (Table 2)	Country	Population	Latitude	Longitude	classification	N	n type mitochondrial DNA			
								les	rid	berg	cf. bed
ROM-13	65	Romania	Nucşcara	45°20'15.54"N	24°46'55.64"E	diploid	8	4	4		
ROM-14		Romania	Saon	45°13'04.79"N	28°32'33.85"E	ridibundus	3	3			
ROM-15	66	Romania	Argineşti	44°34'32.38"N	23°24'47.96"E	diploid	7		7		
ROM-16		Romania	Histria	44°34'21.17"N	28°42'47.34"E	ridibundus	3	3			
ROM-17	67	Romania	Since	44°33'40.00"N	28°45'48.00"E	all-ridbundus	7	7			
ROM-18	68	Romania	Hinova	44°32'23.00"N	22°46'38.00"E	diploid	5	3	2		
ROM-19	69	Romania	Scăpău	44°27'39.24"N	22°43'29.39"E	all-ridbundus	5	5			
ROM-20	70	Romania	Basarabi	44°10'48.00"N	28°24'36.3"E	all-ridbundus	8	8			
ROM-21		Romania	Furnica	43°57'36.67"N	28°00'07.46"E	ridibundus	3	3			
ROM-22		Romania	Mangalia	43°48'59.16"N	28°34'47.11"E	ridibundus	2	2			
BG-1	71	Bulgaria	Durankulak	43°42'2.88"N	28°34'31.80"E	all-ridbundus	9		9		
BG-2	72	Bulgaria	Bolata Dere	43°23'37.68"N	28°27'58.68"E	all-ridbundus	8	8			

A2: Variable sites in the ND2 and ND3 gene and nucleotide composition among 40 haplotypes of *lessonae*-type mtDNA.

Site (position)	ND2																								ND3					
	1	1	2	3	3	4	4	4	4	5	6	6	6	7	7	8	8	8	8	8	9	9	10	10	11	11	11	11	11	12
	1	7	2	6	9	5	5	3	9	0	1	9	2	1	8	7	2	5	0	2	5	6	1	6	2	3	5	8	7	3
L1	G	T	G	C	G	A	A	A	G	C	A	G	C	A	T	A	G	G	G	G	G	A	T	T	A	T	A	T	T	C
L2	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
L3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
L4	.	.	.	T	.	G	.	.	.	.	.	A	.	.	C	G	A	.	.	.	.	.	.	.	.	.	.	.	.	.
L5	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.
L6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.
L7	.	.	.	T	.	G	.	.	.	.	.	A	.	.	C	G	A	.	A	.	.	.	.	.	.	.	.	G	.	.
L8	.	.	.	.	.	.	.	.	.	T	.	A	.	.	.	G	A	.	.	.	.	.	.	.	.	.	.	.	.	T
L9	.	.	.	.	A	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L10	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L11	.	.	.	.	A	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
L12	.	.	.	.	A	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L13	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L14	.	.	.	T	.	G	.	.	.	.	.	A	.	.	C	G	A	.	.	.	.	.	.	.	.	.	.	G	.	.
L15	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L16	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.
L17	.	C	.	T	.	G	.	.	.	.	.	A	.	.	C	G	A	.	.	.	.	.	.	.	.	.	.	G	.	.
L18	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.
L19	.	C	.	T	.	G	.	.	.	.	.	A	.	.	C	G	A	.	.	.	.	.	.	.	.	.	.	.	.	.
L20	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L21	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.
L22	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.
L23	.	.	.	T	.	G	.	.	.	.	.	A	.	.	C	G	A	.	.	.	.	.	.	G	.	.	.	G	.	.
L24	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.
L25	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
L26	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L27	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	G	.	.	.	.	.
L28	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.
L29	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L30	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L31	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.
L32	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L33	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L34	.	.	.	T	.	.	.	.	.	.	.	A	.	.	C	G	A	.	.	.	.	.	.	.	.	.	.	.	.	.
L35	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L36	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.
L37	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	C	.
L38	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.
L39	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L40	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.

A3: Variable sites in the ND2 and ND3 gene and nucleotide composition among 32 haplotypes of *ridibundus*-type mtDNA.

	Site (position) ND2																											ND3					
																										1	1	1	1	1	1	1	1	1	1
		2	2	2	3	3	3	4	5	5	5	6	6	7	7	7	7	7	7	7	8	8	9	9	9	0	0	0	0	1	2	2	2	3	3
	3	2	3	7	2	4	4	7	0	7	9	2	5	1	2	2	3	4	4	6	6	9	6	6	7	0	0	1	2	1	0	8	9	1	1
	4	1	4	3	1	2	5	4	4	9	1	4	2	1	6	8	5	4	7	2	7	4	7	9	8	8	9	5	4	3	6	4	5	4	5
R1	A	G	T	T	T	A	C	C	G	G	G	C	T	C	T	C	C	G	G	G	A	T	G	C	T	A	C	A	C	C	A	C	T	T	G
R2	.	.	.	C	.	G	.	T	.	A	A	T	.	.	.	.	.	.	A	A	.	C	.	.	.	.	.	.	.	.	G	T	.	C	.
R3	.	.	.	C	.	G	.	T	.	A	A	T	.	T	.	.	.	.	A	.	.	C	.	.	.	.	.	.	.	T	.	T	C	C	.
R4	.	.	.	C	.	G	.	T	.	A	A	T	C	.	.	.	.	.	A	A	.	C	A	T	.	.	.	.	.	.	G	T	.	C	.
R5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
R6	.	.	.	C	.	G	.	T	.	A	A	T	.	.	.	.	.	.	A	.	.	C	A	T	C	.	.	.	.	T	.	T	.	C	.
R7	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
R8	C	.	.	C	.	G	.	T	.	A	A	T	.	.	.	.	.	.	A	.	.	C	A	T	C	.	.	.	.	T	.	T	.	C	.
R9	C	.	.	C	C	G	T	T	.	A	A	T	C	.	.	.	.	.	A	A	G	C	A	T	.	.	.	.	.	.	G	T	.	C	.
R10	C	.	.	C	.	G	.	T	.	A	A	T	C	.	.	.	.	.	A	A	.	C	A	T	.	.	.	.	.	.	G	T	.	C	.
R11	C	.	.	C	.	G	.	T	.	A	A	T	.	.	.	.	.	.	A	.	.	C	A	T	.	.	.	.	.	T	.	T	.	C	.
R12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.
R13	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
R14	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
R15	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.
R16	.	.	.	C	.	G	.	T	.	A	A	T	C	.	.	.	.	.	A	A	.	C	A	T	.	.	.	.	.	.	.	.	.	.	.
R17	.	.	.	C	.	G	.	T	.	A	A	T	.	.	.	.	.	.	A	.	.	.	A	T	C	.	.	.	.	T	.	T	.	C	.
R18	.	.	.	C	.	G	.	T	.	A	A	T	.	.	.	.	.	.	A	.	.	C	A	T	C	.	.	.	.	T	.	T	.	C	A
R19	.	.	.	C	.	G	.	T	.	A	A	T	.	.	.	.	T	.	A	.	.	.	A	T	.	.	.	.	.	T	.	T	.	C	.
R20	.	.	.	C	C	G	T	T	.	A	A	T	C	.	.	.	.	.	A	A	.	C	A	T	.	.	.	.	.	.	G	T	.	C	.
R21	.	.	.	C	.	G	.	T	A	A	A	T	.	.	.	.	.	.	A	.	.	C	A	T	C	.	.	.	.	T	.	T	.	C	.
R22	.	.	.	C	.	G	.	T	.	A	A	T	.	.	.	.	.	.	A	.	.	C	A	T	C	.	T	.	.	T	.	T	.	C	.
R23	.	.	.	C	.	G	.	T	.	A	A	T	.	.	C	.	.	.	A	.	.	C	A	T	C	.	.	.	.	T	.	T	.	C	.
R24	.	A	.	C	.	G	.	T	.	A	A	T	.	.	.	.	.	.	A	.	.	C	A	T	C	.	.	.	.	T	.	T	.	C	.
R25	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.
R26	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
R27	.	.	.	C	C	G	T	T	.	A	A	T	C	.	.	.	.	.	A	A	G	C	A	T	.	.	.	.	.	.	G	T	.	C	.
R28	.	.	.	C	.	G	.	.	.	A	A	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
R29	.	.	C	C	.	G	.	T	.	A	A	T	.	.	.	.	.	.	A	.	.	C	A	T	C	.	.	.	.	T	.	T	.	C	.
R30	.	.	.	C	.	G	.	T	.	A	A	T	.	.	.	.	.	.	A	.	.	C	A	T	.	.	.	.	.	T	.	T	.	C	.
R31	.	.	.	C	.	G	.	T	.	A	A	T	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	T	.	T	.	C	.
R32	.	.	.	C	.	G	.	T	.	A	A	T	.	.	.	.	.	.	A	.	.	C	.	.	.	.	.	.	.	T	.	T	.	C	.

Unisexual paternal lineage of triploid water frogs (*Pelophylax esculentus*) from Central Europe

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Abstract.- The edible frog (*Pelophylax esculentus*, genotype LR) is a natural hybrid between the pool frog (*P. lessonae*, LL) and the marsh frog (*P. ridibundus*, RR). It reproduces trough hybridogenesis; i.e. it excludes one of the parental genomes at gametogenesis and produces haploid unrecombined clonal gametes containing the other parental genome. This complex of species represents one of the rare cases of quasi-asexuality (both sexes but transmission of only one genome without recombination) among vertebrates. Moreover some populations shelter triploid specimens.

In order to enlighten the breeding system occurring in populations containing triploids we investigated the population genetics and gamete production pattern of populations nearby Western Carpathian mountains.

We showed that populations from Slovakia are of LE-system breeding type with multiple, and probably still ongoing, primary hybridization events. In this population we found a triploid unisexual paternal lineage constituted of all male triploid LLR frogs. In Eastern Czech Republic we found a zone of sympatry of frogs originating from the LE-system (constituted of only one R hemiclone), the triploid male lineage but also repeated multi locus genotypes betraying the presence of hemiclonally transmitted L genomes characteristic for RE-systems. These situations illustrate the diversity and dynamic of water frog populations systems and highlight the evolutionary creative instability of this peculiar genetic complex.

Key words: Hybridization, polyploidy, asexual, hybridogenesis, hemiclone, *Pelophylax esculentus*

Introduction

Vertebrates that reproduce asexually (i.e. without regularly going through meiotic recombination) represent less than 0.1% of extant animal species (Dawley 1989, Vrijenhoek et al. 1989, Schön et al. 2009). The asexuals arose by hybridization between phylogenetically related sexual species (Vrijenhoek 1989, Neaves and Baumann 2011, Choleva et al. 2012, but see Sinclair et al. 2010). If multiple hybridization events are suspected to have occurred, one should observe the formation of various asexual lineages (Schultz 1973, Alves et al. 2001, Janko et al. 2005). Once established, asexual lineages maintain themselves by adopting one of the three non-sexual reproductive modes: parthenogenesis, gynogenesis and hybridogenesis. The first two mechanisms are producing clonal offspring, while the third one leads to hemiclonal offspring. Parthenogenesis (i.e. the development of unreduced eggs without sperm) and gynogenesis (i.e. embryogenesis triggered by the sperm but without incorporation of the male genome) are completely asexual, producing female offspring only. Hybridogenesis (Schultz 1969) can be considered quasi-asexual because the offspring inherited half of their genomes clonally (usually the maternal one) and the other half from a sexual species, but there is no recombination between the maternal and the paternal genomes, except on rare occasion (Schmeller et al. 2005, Lamatsch and Stöck 2009).

Studies of such clonal and hemiclonal breeding systems can provide insights on the advantages and disadvantages of sexual and asexual reproduction, an issue that is still much debated (Avise 2008). There is consensus that, in the long run, sex is beneficial because it allows the purging of deleterious mutations and enhances genetic diversity upon which selection can act. For the short term, however, asexuality should be superior to sexuality because all-female production saves the costs of producing males (Maynard Smith 1971). As a result of this immediate benefit, asexual taxa should have a demographic advantage over sexual ones and replace them

before they themselves suffer from the negative consequences of clonal reproduction and, hence, the benefits from sexuality would take effect.

Hence, to explain the continuing coexistence of both reproductive modes, we have to assume that sexual reproduction conveys not only long-term but also short-term benefits and, conversely, that asexual reproduction enjoys not only short- but also long-term benefits. The most popular explanation for short-term benefits of sexuality comes from the “Red-Queen-Hypothesis” (Van Vallen 1973, Jaenik 1978, Neiman and Koskella 2009). It assumes that parasites produce short-term fluctuations of the environment that lead to frequency-dependent selection for which genetic diversity is essential (Hamilton 1980, Lively et al. 1990). To explain long-term benefits for asexuals two, not mutually exclusive hypotheses have been proposed: (1) The “General Purpose Genotype” (GPG) hypothesis assumes that interclonal selection has resulted in some clones that are adapted to a broader range of environmental conditions and a more generalized utilization of resources then their sexual relatives (Lynch 1984). For asexual taxa that have arisen from hybridization, which is the case of all vertebrates with asexual reproduction (Dawley 1989), such broad ecological tolerance can also be achieved by merging the genetic setup of two parental species that are adapted to different niches. (2) The “Frozen Niche Variation” (FNV) hypothesis assumes periodical generation of clones – in vertebrates for instance through repeated primary hybridization between two sexual parental species - with each clone carrying only a small proportion of the sexual species’ genetic variability and being adapted to a narrow niche (Vrijenhoek 1979). Selection then favours clonal lineages whose niches overlap little with those of other clones and of their sexual progenitors. Only when all clones are pooled, we get the impression that the asexual taxon succeeds under the whole array of ecological niches. Unfortunately, both hypotheses make the same prediction, namely that asexual taxa should be relatively more abundant under harsh and peripheral environmental conditions. This is consistent with the observation of “geographic parthenogenesis” (Vandel 1928, Lynch 1984, Vrijenhoek and Parker 2009); but it does not allow to decide whether the success of asexuals is due to GPG or to FNV model. For such a test, we need a detailed analysis of the existing clones and whether the same clone(s) occur over a broad

range of ecological niches, as predicted by the GPG hypothesis, or whether each clone is restricted to a narrow niche, as predicted by the FNV hypothesis. In this study, we attempt such a test, for the hemiclonal water frog *Pelophylax esculentus*.

The *Pelophylax esculentus* complex

Pelophylax esculentus (Linnaeus, 1758), the edible frog (genomic composition LR), is a hybrid frog taxon originally stemming from matings between *P. lessonae* (Camerano, 1882), the pool frog (LL), and *P. ridibundus* (Pallas, 1771), the marsh frog (RR). It is perpetuated through hybridogenetic mode of reproduction. Because of this reproductive mode the hybrid needs to live in sympatry with the parental species whose genome has been excluded in order to restore hybridity at each generation. In most of the species' European range one finds the so called LE-systems where the hybrid excludes its *P. lessonae* genome (L genome) passes on its *P. ridibundus* genome (R genome) and generates new hybrids by mating with *P. lessonae* individuals. In some populations, however, the mirror breeding system is found, the RE-system. Here, *P. esculentus* excludes the R, transmit its L genome and mates with *P. ridibundus* to perpetuate the hybrid line.

As this reproductive mechanism involves chromosomes from two different species (Zaleśna et al. 2011), it can create meiotic problems and occasionally produces diploid gametes which after fusion with haploid ones result in triploid individuals (Berger and Roguski 1978, Uzzell et al. 1975). Such triploid *P. esculentus* have been found in several areas of the species' range, with a concentration in Northern Europe around the Baltic Sea (Rybacki and Berger 2001, Plötner 2005). There, the most frequent population structure is one with no parental species and three types of hybrids: diploid LR and triploid LLR and LRR. The genetic structure and breeding system in these so-called EE systems has been well studied in the last decade (Christiansen and Reyer 2009, Arioli et al. 2010, Jakob et al. 2010, chapters 1 and 2 of this thesis). In those all-hybrid populations, triploids of both genomic compositions (LLR and LRR) are usually formed by fusion of diploid clonal LR eggs produced by LR females with haploid recombined L or R sperm of LLR or LRR males respectively. Diploid hybrids (LR) can arise from fusion of haploid

recombined L and R gametes of male and female LLR and LRR, respectively, and from fusion of recombined L eggs of LLR females and haploid clonal R sperm of LR males (for details see Christiansen 2009).

Various studies have reported the existence of triploids and all-hybrid populations also for Central Europe (Berger 1988a, Rybacki and Berger 2001, Mikulíček and Kotlik 2001, Plötner 2005). However, it was largely unknown, how breeding systems function there, i.e. which genotypes and sexes contribute what types of recombined respectively clonal gametes. In this study, we fill this gap for eight populations containing triploids in a region of Western Slovakia and Eastern Czech Republic. Our approach consists of four parts:

(1) Based on microsatellite analysis, we calculated genetic diversity and differentiation within and among populations to investigate the differences respectively similarities between the gene pools of all frog types in all populations.

(2) We determined the types of gametes produced by each frog type and sex, using flow cytometry on the gametes and analyzing the genotypes of offspring produced from artificial crosses between males and females of known genotypes.

(3) We searched for hemiclones (or multi-locus genotype lineages) and investigated whether they are widespread over the whole area, as predicted by the GPG hypothesis, or specific for certain ponds, as assumed by the FNV hypothesis.

4) Finally, we tried to elucidate the origin of certain hemiclones by comparing genetic differentiation between the L and R genomes of various genotypes.

With the combined results from the four approaches, we were able to describe a new breeding system type presenting a unique asexually reproducing male hybridogenetic lineage of triploid water frogs from Central Europe.

Material and methods

Populations

During springs 2008, 2009 and 2010 we sampled a total of 524 specimens from eight populations in Slovakia and one in the Czech Republic (see Table 1 for names, coordinates, frog sample size and type of each population). Frogs were hand-collected at night, kept separated by sexes in spacious plastic containers. They were assigned to taxa (*P. lessonae*, *P. ridibundus* and *P. esculentus*) according to species-specific morphological characters (Berger 1988b, Plötner 2005). All specimens were measured, photographed, and toe clipped. We took blood smears on microscopic slides and, for the frogs selected for crosses, blood samples for latter analysis. Blood smears were used for a first rough on site determination of the ploidy level of *P. esculentus* hybrids by measuring erythrocytes size; triploid erythrocytes are significantly larger than diploid ones (Polls-Pelaz 1988, Vinogradov et al. 1990). Frogs selected for crossing were individually transponded (RFID PIT tag Trovan ID101), separated by sex and population of origin and transported to the University of Zurich. During transport they were stored in cloth bags containing small pieces of rubber sponge and showered daily with fresh water. All frogs survived the journey. After arrival in Zurich they were kept separated by sexes, released in outdoor cages, and fed ad-libitum with live crickets.

Artificial crosses

In order to determine the types of gametes produced by the different hybrids, we originally had planned to cross each hybrid with a least one specimen of each parental species and with another hybrid. In this way, we also wanted to avoid that certain gamete types were overlooked because their existence was masked by genetic incompatibilities of certain combinations and, hence, inviability of the zygotes. However, due to limited egg availability in some females, this planned complete crossing design could not be achieved for all frog types in the populations of Šajdíkove and Borovec (see results in Table 2). Artificial fertilizations were achieved following the Berger et al. (1994)

protocol with the following slight modifications: To induce ovulation, females were injected with 100 µl per 10 gr body mass of a 20 mg/l LHRH hormone in Holtfreter solution (59 mM NaCl, 0.7 mM KCl, 0.9 mM CaCl2, 2.4 mM NaHCO3 and 1.6 mM MgSO4 mM, pH 7.4). Males were anesthetized in a buffered solution of MS-222 (0.15 g/l, pH 7.4) before having one of their testes removed and lacerated into a Petri dish to obtain the sperm solution. This protocol permits the use of the same sperm solution to fertilize eggs from different females, and to cross the same female with different males. After about 15 days the obtained embryos reached free swimming stage (stage 25, Gosner 1960) and were euthanized using an overdosed MS-222 buffered solution (2 g/l, pH 7.4). The offspring of a few crosses were used for other experiments (Pruvost et al. 2013) but their genotypic data could also be use for our purpose.

Flow cytometry

Forty three hybrids were analyzed by flowcytometry to confirm their ploidy level and, if males, to determine ploidy level of their sperms. Blood and sperm samples were stabilized in buffer (475 mM D-(+)-glucose, 40 mM trisodium citrate, 5% dimethyl sulphoxide, pH 7.6) and immediately frozen at -80ºC. Samples of both parental species were used as a diploid standard. A relative nuclear DNA content was measured using DAPI fluorochrome applying a commercial kit Cystain 2 Step High Resolution DNA Staining (Partec GmbH, Germany). Fluorescence intensity of 5000 stained nuclei was measured in Partec PA II flow cytometer with a speed 0.5 µL/sec. Flow cytometric histograms were evaluated using Partec FloMax 2.52 software.

Microsatellite genotyping

DNA was extracted from toe or tail tips of the adult frogs or tadpoles, respectively, stored in 80% ethanol. The Qiagen BiosprintTM 96 DNA Blood Kit was used for extraction following supplier’s protocol.

We used a set of 18 microsatellite primer pairs which were run in four primer mixes:

• Primer Mix 1A - CA1b6, Ga1a19 redesigned (Arioli et al. 2010), RlCA1b5, RlCA5 (Garner et al. 2000), Rrid064A (Christiansen and Reyer 2009)
• Primer Mix 1B - Re2CAGA3 (Arioli et al. 2010), Res16, Res20 (Zeisset et al. 2000), RlCA2a34 (Christiansen and Reyer 2009)
• Primer Mix 2A - ReGA1a23, Rrid169A, Rrid059A redesigned (Christiansen and Reyer 2009), Res22 (Zeisset et al. 2000), Rrid013A (Hotz et al. 2001)
• Primer Mix 2B (PM2B): Re1CAGA10 (Arioli et al. 2010), RlCA18 (Garner et al. 2000), RlCA1a27, Rrid135A (Christiansen and Reyer 2009).

Details on PCR protocols are given by Christiansen (2009) and Christiansen and Reyer (2009, 2011). Fragment length analysis of the PCR products were run on an ABI 3730 Avant capillary sequencer with internal size standard (GeneScan-500 LIZ) and the alleles were scored with the GeneMapper software v3.7 (Applied Biosystems).

Loci Res20, RlCA1a27 and RlCA18 were species-specific for *P. lessonae* while loci Re2CAGA3, Res22, Rrid169A and Rrid135A were specific for *P. ridibundus*.

The other 11 microsatellite loci amplified in both the L- and R-genomes. For those loci species-specificities of the alleles were known from previous studies (Christiansen 2005, Christiansen 2009, Arioli et al. 2010, chapters 1 and 2 of this thesis).

Estimation of null alleles and selection of microsatellite loci

The 18 microsatellites loci were used to determine and/or confirm the genomic composition of the crossed specimens and their offspring in terms of taxa and ploidy level. However, given that *P. lessona*e genome (L) and *P. ridibundus* genome (R) do not recombine, these two genome types have to be considered separately in the genetic analyses. Samples which were not amplified during the first analysis were re-run for PCR two to three times. When even then no allele was amplified, we attributed this to the presence of a null allele. Prior to any analysis of the microsatellite dataset we tested for the presence of null alleles. For this test, we had to use two different methods, as our sample contained specimens of the two parental species and of hybrids with different ploidy. For the parental subpopulations we tested -

separately for LL and RR - for potential genotyping errors like stuttering, allelic drop-out or presence of null alleles using the program Micro-Checker version 2.2.3 (Van Oosterhout et al. 2004). In case of heterozygote deficits, only null alleles were inferred as the most likely cause. We estimated their frequencies using the Brookfield 2 null allele estimator which treats non-amplifications as data and regards them as null homozygotes when calculating null allele frequencies (Brookfield 1996). This method cannot be applied to the diploid hybrids. For these frogs, we visually inspected the L and then R genome, and considered the absence of an allele as evidence for a null allele. We then excluded any loci showing an estimated null allele frequency greater than 0.2 in any of the populations. This led us to exclude loci RlCA5 and Res16 for the analyses of both genomes. We also had to exclude Re1CAGA10 for the L genome and locus RlCA2a34 for the R genome analyses. Loci Ga1a19redesigned, Rrid064A and Rrid059Aredesigned in the L genome, and locus ReGA1a23 in the R genome, appeared to be monomorphic. Therefore, these loci were also excluded from the analyses of the respective genomes. This left us with 8 loci for the L genome and 11 for the R genome:

• CA1b6, RlCA1b5 and Rrid013A for both genomes

• Res20, RlCA2a34, ReGA1a23, RlCA1a27 and RlCA18 for the L genome only

• Ga1a19redesigned, Rrid064, Re2CAGA3, Res22, Rrid169A, Rrid059Aredesigned, Re1CAGA10 and Rrid135A for the R genome only.

Genetic diversity and differentiation

We calculated the gene diversity corrected for sample size, expressed by the expected heterozygosity (He) according to Nei (1978) and global, as well as pair-wise, fixation index (F_{ST}) according to Weir and Cockerham (1984) with permutation tests. For this we used the program SPAGeDi version 1.3 (Hardy and Vekemans, 2002) which allows the combination of multiple ploidy levels in the same analysis. The calculated F_{ST} can be negative when levels of differentiation are close to zero, indicating no population differentiation at these loci (Weir and Cockerham 1984). In such cases, we assigned a value of zero to negative F_{ST} values. To interpret F_{ST} values we followed Wright's

guideline suggesting that a value lying in the range 0–0.05, 0.05-0.15, 0.15-0.25, and above 0.25 indicates little, moderate, great, and very great genetic differentiation, respectively (Wright 1978; Hartl and Clark 1997). To visualize genetic differentiation, respectively similarity, between genotypes and populations, we performed separate cluster analyses for L and R genomes (based on pairwise F_{ST} values), using the Euclidian distance metric and the complete linkage aggregation criteria implemented in the software PermutMatrix (Version 1.9.3, Caraux and Pinloche 2005). Concerning genetic diversity we used two tailed pair-wise t-tests on the values of He for each locus in order to test the significance of differences between different frog types, independent of origin. All statistical tests were run using the program R (version 2.15.1, R Development Core Team 2012).

Hemiclonal diversity

As coined by Vrijenhoek et al. (1977) the term “hemiclone” refer to the clonally transmitted haploid genome, which in our case can be of the L or R type. It is determined by a multi locus genotype (MLG), defined by the identical combination of alleles found in our microsatellite loci analysis and recognized as a hemiclone when present in our sample more than three times.

As different hemiclonal gametes may fuse (syngamy) on the basis of hybrid x hybrid mating, we also searched for possible hemiclonal combinations in the entire genome of the parental species (LL and RR) as well as that of the diploid and triploid hybrids (LR and LLR). To do this we used the Excel add-in GenAlEx version 6.4 (Peakall and Smouse, 2006) which concatenates the alleles of the considered loci and then compares this combinations in order to find similar MLGs. We performed an exhaustive search for all MLG types (L, R, LL, RR, LR, and LLR) in all frog genotypes by searching for:

• L hemiclones in LR specimens based on 10 loci (CA1b6, RlCA1b5, Ga1a19redesigned, Rrid064A, Res20, RlCA2a34, ReGA1a23, Rrid013A, RlCA1a27, and RlCA18)

• L+L hemiclone combinations in LLR and LL specimens based on the 10 loci cited above

• R hemiclones in LR and LLR specimens based on 12 loci (CA1b6, RlCA1b5, Ga1a19redesigned, Rrid064A, Re2Caga3, Res22, ReGA1a23, Rrid169A, Rrid013A, Rrid059Aredesigned, Re1CAGA10, and Rrid135A)
• R+R hemiclone combinations in RR specimens based on the 12 loci cited above
• L+R hemiclone combinations in LR specimens based on 14 loci (CA1b6, RlCA1b5, Ga1a19redesigned, Rrid064A, Res20, Re2Caga3, Res22, ReGA1a23, Rrid169A, Rrid013A, Re1CAGA10, RlCA1a27, RlCA18, and Rrid135A)
• LL+R hemiclone combinations in LLR specimens based on the same 14 loci as for the LR MLG - with the difference that, in the case where the two L alleles where different, we had to fuse them in a new fictive one (using the sum of the two alleles sizes). When found, hemiclones were named according to the following rules: Hemiclone type (L, R or LL) followed by a capital letter attributed in accordance to descending overall frequency (e.g. L-A = *P. lessonae* hemiclone-A = most frequent L hemiclone).

Results

Population composition

PCR runs successfully amplified the 18 microsatellites and allowed us to determine the genomic composition of the 524 sampled specimens. With the exception of the Šprinclov Majer locality, where we found only *P. ridibundus* (RR), all other populations contained two or three genotypes. Based on their composition, they were classified into four population types (PT1-4), each represented by two localities:

PT1: LL and LR frogs (Borský Mikuláš and Kalaštov)
PT2: LL, LR and RR frogs (Brodské and Šaštin-Stráže)
PT3: LR and LLR frogs (Bahno and Kozi Chrbát)
PT4: LR, LLR and RR frogs (Šajdíkove Humence and Borovec).

All triploid LLR specimens sampled in the four populations of PT3 and PT4 happened to be only males (N=83). Their LL genomes showed the exact same genotype for all loci expressing L alleles, with one minor dinucleotide

repetition dissimilarity in the only sampled Czech population (Borovec): here locus RlCA18 amplified alleles 177 and 181, while all LLR frogs from Slovakia carried alleles 179 and 181.

Gamete production

Flow cytometry

Flow cytometric analysis allowed to distinguish between different ploidy levels (diploids, triploids and one tetraploid individual) and between parental genotypes (RR and LL), but did allow to distinguish between diploid hybrids (LR) and parental species (flow cytometric histograms overlapped). Similarly, haploid and diploid sperms revealed clear non-overlapping pattern. Parental and diploid males produced haploid sperms, and LLR males produced diploid sperms. Sperm samples of LLR males were clearly different from RR blood samples, showing that triploid males do not produce RR sperms. However, based on flow cytometric histograms it was not possible to distinguish if LLR males produce LL or LR sperms. One tetraploid LLRR male produced predominately haploid sperms and low amount of diploid cells of unknown genotypic composition.

Artificial crosses

We genotyped 2’216 offspring from 96 crosses through microsatellite analyses. Knowing the genotypes of the parents we were able to unambiguously determine the type of gametes they produced in term of specificity and of ploidy.

All specimens of the parental species used for the crosses acted as normal haploid gamete donors with chromosome segregation in accordance to the second Mendel’s law. Results of the gametes produced by the hybrid frogs are shown in Table 2.

LR hybrids, of both sexes, from all the studied populations always gave haploid R gametes. The triploid LLR hybrids males from the four PT3 and PT4 populations always produced diploid LL gametes. Without any exception these gametes clonally transmitted the exact two same LL genomes to the offspring. The one tetraploid specimen found in Kozi Chrbát (WFB015-54, genotype LLRR) gave haploid R gametes.

Population genetics

Genetic diversity and differentiation

The genetic diversity estimates (H_e), for the L genomes (H_{eL}) and for the R genomes (H_{eR}), are presented in Table 3 (with details for each locus in Appendix A1).

Values for each frog types, independent of its origin (i.e. LL, LR, LLR and RR respectively, pooled over all populations), show for the L genome significant differences in genetic diversity between LLR and both LL frogs and LR frogs (mean difference for LL: 0.384 ±0.096, $t_{(7)}$=2.364, p=0.005; m.d. for LR: 0.352 ±0.102, $t_{(7)}$=2.364, p=0.011) and no difference between LL and LR frogs (m.d.=0.032 ±0.017, $t_{(7)}$=2.364, p=0.111). For the R genome, differences are significant between RR and both, LR and LLR (m.d. for LR: 0.217 ±0.048, $t_{(10)}$=2.228, p=0.001; m.d. for LLR: 0.218 ±0.063, $t_{(10)}$=2.228, p=0.006), but not between LR and LLR frogs (m.d.: 0.001 ±0.035, $t_{(10)}$=2.228, p=0.996).

Global F_{ST} values show significant and substantial differentiation among population for both genomes. The mean values of F_{ST} were 0.271 for the L genome and 0.114 for the R genome, assigning 27.1% and 11.4%, respectively, of the genetic variation to inter-population differences.

Pairwise F_{ST} values for the comparisons among frog types, regardless of their population of origin, are given in Table 4. For the L genomes we found little genetic differentiation between LL and LR frogs (F_{ST} =0.021), but very large differentiation between LLR and both, LL and LR frogs (F_{ST} =0.388 and 0.361 respectively). For R genomes, the genetic differentiation is small between LR and LLR frogs (F_{ST} =0.019), while it is large between RR and both, LR and LLR frogs (F_{ST} =0.133 and F_{ST} =0.129, respectively).

F_{ST} values for pairwise comparisons between all frog types from each population are given in the Table 5 and illustrated in Figure 2. With respect to the L genomes, pairwise F_{ST} values clearly separate triploid LLR hybrids from LR and LL frogs, as indicated by the light green areas in the matrix of Table 5 and the distinct LLR cluster in Figure 2. Hence, these triploids (all males) in population types PT3 and PT4 are genetically not only strongly differentiated from the parental LL frogs in population types PT1 and PT2, but also from the sympatric diploid LR in their own populations. In contrast, there is little to only

moderate genetic differentiation between parental LL frogs and diploid LR hybrids from both the same (PT 1 and PT2) and other populations types (PT3 and PT4). This is obvious from the darker green matrix fields in Table 5 and the joint cluster of LL and LR in Figure 2. The only exception are diploid LR from the Czech population of Borovec which are genetically distinct from all genotypes carrying an L genome in all other populations (see light green row for Boro-LR in Table 5 and separate cluster in Figure 2).

Concerning the R genomes, parental RR individuals form their own cluster (Figure 2) with mostly little to large genetic differentiation between them, and mostly moderate to large differentiation between them and both diploid and triploid hybrids from all population types (Table 5). In contrast, there is only little to moderate differentiation among R genomes of both hybrid types (LR and LLR) from all populations (Table 5). As a result, for the R genome the diploid LR hybrids cluster with triploid LLR hybrids: Again, the Czech population from Borovec stands out, because both the diploid and the triploid hybrids are genetically clearly distinct from hybrids and parental RR in all populations.

Overall, these results indicate that diploid *P. esculentus* hybrids (LR) receive their haploid L genome from frogs of the parental species *P. lessonae* (LL), rather than from triploid LLR hybrids. This is consistent with the above results from gamete and offspring type analyses which showed that LLR males produce diploid LL, rather than haploid L sperm (Table 2). In contrast, the clustering of LR with LLR with respect to the R genome suggests an exchange of R between the two hybrid types. This must be in the direction of LR to LLR, because only diploids produce haploid R gametes (Table 5). Details of the results and the special situation in the Czech population of Borovec will be dealt with in the Discussion.

Hemiclonal diversity:

The results of the MLG analyses are presented by hemiclone type in Table 6.
With respect to the R genomes, we detected a total of 14 hemiclones with different frequencies among populations. In the Czech population of Borovec we found only a single hemiclone (R-B), whereas all Slovak populations contained multiple R hemiclones, ranging from four in Brodské to eight in

Šaštin-Stráže. Hemiclone R-A occurred in all four population types (PT1-4); five (R-F, R-H, R-K, R-L and R-N) occurred only in populations with parental LL frogs (PT1 and/or PT2); and three hemiclones (R-B, R-G, and R-M) were found only in populations with triploid LLR hybrids (PT3 and PT4). The remaining four hemiclones (R-C, R-E, R-I and R-J) were neither universal nor specific for certain population types.

Concerning the L genome, the number of hemiclones was much smaller. We detected only a single L hemiclone (L-A) and two LL “bi-hemiclones” (LL-A and LL-B). L-A occurred only in diploid hybrids from Borovec, but there in a very high proportion (38 out of 50 sampled LR frogs), whereas the two “bi-hemiclones” were present in all triploid hybrids. One of them (LL-B) was also restricted to Borovec, the other one (LL-A) was present in the three Slovak populations of Bahno, Kozi Chrbát and Šajdíkove). However, it is important to note that those two bi-hemiclones differ only by one allele, showing one dinucleotide repetition difference on the locus RlCA18.

Based on the above knowledge of hemiclone types within populations, we searched for the presence of specific hemiclone combinations within the whole genome of the specimens. Among diploid frogs from all populations, a combined MLG lineage was only found in Borovec (Comb-A). This combination of hemiclones L-A and R-B was present in 35 out of 50 LR frogs. In triploid specimens from population types PT3 and PT4, the variety was higher. In the Slovak populations of Kozi Chrbát, Šajdíkove and Bahno six hemiclone combinations were detected, all resulting from the combination of one unique LL hemiclone (LL-A) with different R hemiclones (Table 6).

Discussion

Our study of gamete production patterns and genetic differentiation among populations of water frogs within a relatively small geographical area revealed strong genetic structuring and the existence of different breeding systems. Depending on the genomic composition of the populations, pools of the two parental species genomes interact differently in the hybrids, affecting the types of gamete they produce.

Genetic structure

The haploid R gamete production by LR hybrids and the existence of R, but no L hemiclones (with one exception in Borovec), betrays the signature of an LE breeding system, where diploid hybrids persist though the fusion of haploid clonal R gametes from *P. esculentus* with recombined haploid L gametes from *P. lessonae*. This interpretation is further confirmed by our calculation of F_{ST} values: with respect to the L genome, genetic differentiation is small between LR and LL (mean F_{ST}=0.039, Table 4), and the two genotypes are grouped together in the same cluster for all populations of the PT1 and PT2 type (Figure 2a). In contrast, with respect to the R genome, hybrid and parental specimens are more differentiated (mean F_{ST}= 0.133, Table 4), and LR and RR from all populations of the PT2 and PT4 type appear in different clusters (Figure 2b). These results suggest a repeated flow of L genomes from *P. lessonae* to diploid *P. esculentus*, whereas the pool of clonal R genomes contained in the LR hybrids is differentiated from the one contained in the parental RR frogs. Thus, despite of their sympatry, RR are less similar to LR in the same population than to RR in other populations which were up to 55 km away.

In populations containing triploid LLR hybrids (PT3 and PT4), the gamete production pattern, the hemiclone situation and the F_{ST} values consistently indicate a “frozen nature” of a double L genome. In these populations, triploid frogs (which were all males) conveyed the same double L MLG lineage. Interestingly, all triploid frogs from the three Slovak populations carried the exact same two L genomes, while the one from the Czech

population of Borovec, 130 km away, only differed by one allele at the locus RlCA18. This difference is most probably caused by post-lineage formation mutation.

With respect to the R genome, triploid LLR hybrids result from the fusion of LL sperm produced by LLR males and haploid R eggs, predominantly from LR females. This follows from both, results of the gamete production study and the lower genetic differentiation in the R genome between LLR and LR than between both hybrid types and the parental species RR (see Table 5 and clustering in Figure 2b). This higher contribution, of the LR frogs to the genetic R pool of the LLR frogs, compared to RR, is also confirmed by the fact that in the Slovak populations the most frequent R hemiclones occur in both diploid and triploid hybrids.

Origin of hemiclones

The existence of only one LL hemiclone in all three Slovak populations and one that differs by only one dinucleotide repetition in Borovec suggests a single origin of the LL MLG lineage. Given the high genetic differentiation in L genomes between the LLR and the group of Slovak LL and LR frogs (Figure 2a), this origin is unlikely to have been in-situ. It is probably due to migration of a single MLG lineage from other areas, with one mutation occurring on the way between the Czech and the Slovak populations. Our data do not allow tracing back the origin of this double hemiclone. It may have originated in the Czech region, but it also may be the result of migration from a more distant place. The latter is probably also true for the single L hemiclone found in Borovec. The single R hemiclone of Borovec is also found, in lower proportion, in the Slovak populations. The existence of a much higher number of R hemiclones in the Slovak populations is a hint that in this region primary hybridization between *P. lessonae* and *P. ridibundus* might still be going on where the respective taxa live in sympatry (PT2) or a least in very close proximity. Such multiple endemic origin of hybrid hemiclones has also been documented for other populations (Tunner 1974, Uzzell and Berger 1975, Hotz et al. 2008). It permits a dynamic clonal turnover ensuring the genetic diversity necessary for their ecological success. However, to really identify the

populations where the detected L, LL and R hemiclones originated from, one would need to extend the research area.

Breeding systems

In population types PT3 and PT4, the triploid males manage to maintain themselves by transmitting their double L genomes and by using the R genomes of the diploid LR frogs from a typical LE-system. This is why we conclude that the Slovak triploid populations are equivalent to an L-E system with the addition of triploid LLR frogs and that we decided to name such system “modified LE-system”. This system illustrates a modification of the hybridogenetic mechanism of genome exclusion depending on a genome dosage effect altering the “decision” as to which genome is excluded and which is propagated.

The unique LLRR frog we found was similar to LR in that it eliminated the L genomes and produced haploid R gametes.

A remaining puzzle is where the diploid LR hybrids in these modified LE systems come from. Their production requires haploid L gametes which apparently are not produced in PT3 and PT4 populations. There are a number of possible explanations that are not mutually exclusive, and different ones may apply to different populations.

1. The haploid L gametes may come from LL frogs occurring in small numbers within the pond and/or in larger numbers in nearby ponds. Actually in the Slovak region populations Borský Mikuláš and Šaštin-Stráže the F_{ST} values, attesting of a very low differentiation of the L genomes, shows that they probably act as reservoir of *P. lessonae* specimens necessary to the re-formation of diploid LR at each breeding season.

2. Diploid LR and/or triploid LLR may occasionally produce haploid L gametes. Evidence for such variability in gamete production of the same individual comes from all-hybrid populations in Sweden, consisting of diploid LR and triploid LLR and LRR in both sexes (EE-system; Arioli et al. 2010). Usually, triploids of both sexes exclude the rarer genome and produce haploid gametes of the remaining genome (i.e. L in LLR and R in LRR); diploid males produce haploid R sperm, whereas diploid females produce LR eggs. But

some triploid individuals of both sexes produce also small proportions of diploid gametes (LL or RR), some diploid females produce low numbers of haploid eggs (R), and some diploid males occasionally produce L, rather than R, as well as LR sperm.

3. A third potential explanation may apply to the special situation in the Czech population of Borovec, where we detected an L hemiclone in 76% of the LR frogs sampled (38 out of 50). The existence of this frozen L MLG lineage betrays the hybridogenetic mode of L genome transmission. This is characteristic of a RE-system where diploid LR hybrids discard the R genome during gamete production, clonally transmit the L genome and restore hybridity by mating with RR. Despite the fact that we haven't found such clonal L gamete donors in our crossing experiment, it seems plausible to assume that Borovec is a place where RE- and modified LE-breeding systems meet. According to Ragghianti et al. (2007), RE-systems occur 400 km downstream the river Odra, and it is not unlikely that the whole river basin is colonized by frogs from such a system. If true, the monomorphic L lineage in the modified LE system of Borovec might have arisen from an RE system in the same area.

Evolutionary potential, problems and solutions

The detection of an allotetraploid LLRR specimen with the double LL genome also found in triploid males and a duplicate R genome of the hemiclone lineage R-C briefly opened a potential long-term escape from the genetic dilemma. Such genomic composition would allow recovery of normal meiosis resulting in diploid LR gametes; and this could lead to speciation via tetraploidy (Cunha et al. 2008). This, however, did not happen here. Rather than returning to normal meiosis, the tetraploid returned to the hybridogenetic mode of reproduction typical for LR, i.e. it excluded the L genomes and produced predominantly haploid R gametes. This phenomenon highlights the genomic dosage effect leading to the susceptibility or resistance to exclusion of the two parental genomes.

The difference in gamete production patterns between EE systems from Northern Europe where triploids usually arise from diploid eggs of diploid

females and haploid sperm of triploid males, and the here studied modified LE systems where triploids result from haploid eggs and diploid sperm, is attesting to a polyphyletic origin of the triploids in these two systems. However, at the scale of this region west of the Carpathians, the uniqueness and similarity of the double L genome transmission by LLR males is strong evidence for a monophyletic origin of the triploids in this area. This illustrates the importance of a rare event (in this case area-specific genome segregation differences at meiosis) which can have strong evolutionary implications. Quattro et al. (1992) demonstrated in freshwater fishes of the *Poecilliopsis monacha-occidentalis* complex that a single unisexual hemiclonal lineage colonized a whole region and achieved a substantial evolutionary age (120 000 to 300 000 generations). The Amazon Molly, *Poecilia formosa*, a small all-female live-bearing fish species occurring in fresh water in Mexico, has an estimated age, calculated on the basis of mitochondrial and nuclear sequences, of 280,000 years and approximately 800,000 generations (Lampert and Schartl 2008). The oldest described unisexual vertebrate taxon reproducing hemiclonally is the *Ambystoma* salamander with an estimated monophyletic origin going back to the Pliocene, five million years ago (Bi and Bogart 2010).

In Central European water frogs, a change in meiosis and gamete production instantaneously generated a triploid unisexual paternal lineage that apparently is able to successfully maintain itself and even to spread through a relatively large region. But is there any evolutionary future for the resulting modified LE-system of population types PT3 and PT4? It is important to note that in these populations, the LLR frogs are acting as sink for the R genome stolen form their LR congeners (see LR/LLR cluster in Figure 2b) which are themselves acting as a sink for the L genomes stolen from the sympatric *P. lessonae* frogs (see LR/LL cluster in Figure 2a). In other words, the LLR frogs are sexual parasites on the LR frogs which are, already, sexual parasites on the *P. lessonae* frogs of the population (Schmidt 1993, Joly 2001, Lehtonen 2013). The evolutionary potential of those triploid males mostly lie in their ability to perpetuate themselves and to consist of one of the rare unisexual paternal lineage describe among vertebrates (Dawley 1989, Bogart 2003, Neaves and Baumann 2011). Their existence and perpetuation is a

proof of their ecological and evolutionary success and an argument supporting the General Purpose Genotype model (GPG). While the presence, in the Slovak populations, of a relatively great number of hemiclones is an argument supporting the alternative, but not exclusive model of the Frozen Niche Variation (FNV). As stated by Petit et al. (1999) the success of such a lineage can probably be explained by the hybrid-superiority hypothesis of Moore (1977), who postulated that hybrid populations can be maintained if hybrids are more fit than their parental phenotypes in some environments. Overall, adopting these two different strategies (GPG and FNV), hybrid water frogs of the *P. esculentus* complex managed to escape the predicted inexorable accumulation of deleterious mutation. Hence, genetic mechanisms alone are not sufficient to explain the existence of modified LE-systems. But this is also true for the normal LE-systems of the PT1 type; and yet, they are widespread and apparently successful.

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Tables

Table 1: Populations and number of frogs studied in each of them (N LL = number of *P. lessonae*, N LR = number of diploid *P. esculentus*, N LLR = number of triploid LLR *P. esculentus*, N RR = number of *P. ridibundus*).

Country	*Population*	*Latitude/Longitude*	*N LL*	*N LR*	*N LLR*	*N RR*	*N total*	*Pop. Type*
Slovakia	Bahno	48°37'33"N / 17°16'24"E	-	31	5	-	36	PT3
	Borský Mikuláš	48°37'45"N / 17°11'17"E	15	24	-	-	39	PT1
	Brodské	48°41'37"N / 17°00'29"E	4	35	-	52	91	PT2
	Kalaštov	48°37'55"N / 17°15'12"E	3	32	-	-	35	PT1
	Kozi Chrbát	48°37'53"N / 17°17'41"E	-	20	52	-	72	PT3
	Šajdíkove Humence	48°38'34"N / 17°16'54"E	-	12	20	2	34	PT4
	Šaštin-Stráže	48°37'55"N / 17°08'40"E	27	79	-	26	132	PT2
	Šprinclov Majer	48°12'59"N / 17°11'15"E	-	-	-	10	10	-
Czech Republic	Borovec	49°38'08"N / 18°06'01"E	-	50	6	19	75	PT4
	Total		49	283	83	109	524	

Table 2: Genomic composition and ploidy of the produced gametes

Population	*Genomotype*	*Sex*	*Ind. Num.*	*N cross*	*N offspring*	*Gametes produced (%)*	
Borovec	LLR	M	WFB005-48	3	10	100 LL	
	LR	F	WFB005-41	5	198		100 R
			WFB005-45	3	100		100 R
			WFB005-47	5	156		100 R
		M	WFB005-52	3	106		100 R
			WFB005-55	3	76		100 R
Kozi Chrbát	LLRR	M	WFB015-54	2	32		100 R
	LLR	M	WFB015-55	5	178	100 LL	
			WFB015-56	2	11	100 LL	
			WFB015-57	2	25	100 LL	
			WFB021-16	3	7	100 LL	
			WFB021-17	3	24	100 LL	
			WFB021-18	3	16	100 LL	
	LR	F	WFB021-24	5	104		100 R
			WFB021-30	3	97		100 R
Šajdíkove	LLR	M	WFB007-93	4	86	100 LL	
			WFB008-14	3	93	100 LL	
			WFB015-13	4	14	100 LL	
	LR	F	WFB007-91	2	30		100 R
		M	WFB007-90	2	9		100 R
Šaštin-Stráže	LR	F	WFB007-33	1	8		100 R
			WFB007-35	1	12		100 R
			WFB007-37	4	142		100 R
			WFB015-72	8	284		100 R
			WFB015-73	7	161		100 R
		M	WFB007-52	4	101		100 R
			WFB007-54	5	79		100 R
			WFB015-03	6	84		100 R
			WFB015-04	4	133		100 R
			WFB015-06	7	254		100 R

Table 3: Gene diversity corrected for sample size, Nei 1978 (H_e) for *P. lessonae* genomes (H_{eL}) and *P. ridibundus* genomes (H_{eR}) in the different frog genotypes (LL, LLR, LR, RR). Sample size is given in brackets.

		H_{eL}			H_{eR}		
Population type	Population name	LL	LLR	LR	LLR	LR	RR
	All populations	**0.640** (49)	**0.256** (83)	**0.608** (283)	**0.413** (83)	**0.414** (283)	**0.631** (109)
PT1 (LL + LR)	Borský Mikuláš	**0.650** (15)	**-**	**0.586** (24)	**-**	**0.385** (24)	**-**
PT1 (LL + LR)	Kalaštov	**0.600** (3)	**-**	**0.574** (32)	**-**	**0.418** (32)	**-**
PT2 (LL + LR + RR)	Brodské	**0.594** (4)	**-**	**0.577** (35)	**-**	**0.436** (35)	**0.656** (52)
PT2 (LL + LR + RR)	Šaštin-Stráže	**0.618** (27)	**-**	**0.558** (79)	**-**	**0.396** (79)	**0.602** (26)
PT3 (LLR + LR)	Bahno	**-**	**0.278** (5)	**0.590** (31)	**0.436** (5)	**0.425** (31)	**-**
PT3 (LLR + LR)	Kozi Chrbát	**-**	**0.252** (52)	**0.495** (20)	**0.424** (52)	**0.429** (20)	**-**
PT4 (LLR + LR + RR)	Šajdíkove Humence	**-**	**0.256** (20)	**0.536** (12)	**0.414** (20)	**0.275** (12)	**0.439** (2)
PT4 (LLR + LR + RR)	Borovec	**-**	**0.273** (6)	**0.225** (50)	**0.115** (6)	**0.029** (50)	**0.496** (19)
RR	Šprinclov Majer	**-**	**-**	**-**	-	**-**	**0.549** (10)

Table 4: Pairwise F_{ST} between L (below the diagonal) and R (above the diagonal) genomes, pooled over all genotypes. Global F_{ST} values are given in the left hand corner.

F_{ST}	R:0.114	LL	LLR	LR	RR
L:0.271					
LLR		0.388	x	0.019	0.129
LR		0.021	0.362	x	0.133

Table 5: Matrix of F_{ST} for all 21 frog types x population combinations, for L (greenish, below the diagonal) and R (reddish, above the diagonal) genomes. Darker colors correspond to lower F_{ST} values.

	Bors LL	Kala LL	Brod LL	Sast LL	Bahn LLR	Kozi LLR	Sajd LLR	Boro LLR	Bors LR	Kala LR	Brod LR	Sast LR	Bahn LR	Kozi LR	Sajd LR	Boro LR	Brod RR	Sast RR	Sajd RR	Boro RR	Spri RR
Bors LL	X																				
Kala LL	.108	X																			
Brod LL	.070	.106	X																		
Sast LL	.009	.052	.043	X																	
Bahn LLR	.280	.492	.470	.788	X	.000	.000	.273	.003	.035	.003	.000	.000	.000	.000	.745	.089	.104	.060	.130	.144
Kozi LLR	.423	.592	.584	.403	.000	X	.000	.208	.085	.045	.109	.085	.004	.000	.098	.424	.143	.177	.132	.180	.200
Sajd LLR	.366	.571	.557	.348	.000	.000	X	.220	.035	.093	.040	.016	.000	.000	.025	.544	.137	.152	.106	.171	.195
Boro LLR	.291	.507	.490	.289	.111	.111	.111	X	.312	.302	.252	.258	.193	.245	.423	.181	.197	.247	.537	.185	.360
Bors LR	.022	.166	.067	.000	.340	.478	.427	.349	X	.150	.049	.048	.054	.063	.052	.585	.166	.173	.154	.215	.237
Kala LR	.102	.127	.135	.100	.302	.432	.381	.313	.127	X	.194	.185	.045	.028	.220	.544	.154	.176	.251	.203	.182
Brod LR	.045	.130	.023	.024	.325	.455	.403	.334	.035	.086	X	.038	.062	.093	.063	.492	.144	.142	.120	.186	.207
Sast LR	.066	.112	.097	.015	.318	.417	.371	.325	.031	.104	.030	X	.061	.084	.059	.431	.162	.156	.149	.206	.222
Bahn LR	.077	.000	.076	.041	.315	.450	.397	.326	.079	.089	.069	.071	X	.000	.054	.448	.140	.154	.146	.155	.201
Kozi LR	.142	.159	.122	.112	.450	.560	.523	.460	.116	.213	.125	.140	.127	X	.070	.566	.137	.165	.130	.174	.197
Sajd LR	.113	.036	.072	.087	.396	.515	.482	.410	.140	.029	.077	.105	.041	.147	X	.747	.215	.232	.231	.255	.329
Boro LR	.392	.528	.535	.354	.709	.696	.702	.708	.431	.419	.389	.370	.397	.537	.487	X	.346	.445	.881	.413	.667
Brod RR																	X	.025	.079	.077	.044
Sast RR																		X	.118	.103	.058
Sajd RR																			X	.180	.191
Boro RR																				X	.168
Spri RR																					X

differentiation	L	FST	R
little		0 - 0.05	
moderate		0.05 - 0.15	
great		0.15 - 0.25	
very great		> 0.25	

Table 6: Multi Locus genotypes (MLGs) found in the study and their occurrence across populations.

MLG type	MLG name	Repartition	Pop. type	N Tot.
R (among LR and LLR)	R-A	17 Sast (17 LR), 13 Kozi (9 LLR, 4 LR), 10 Sajd (4 LLR, 6 LR), 6 Bahn (2 LLR, 4 LR), 5 Brod (LR), 3 Bors (LR), 2 Kala (LR)	PT1-4	56
	R-B	50 Boro (3 LLR, 47 LR), 2 Bahn (LR), 1 Kozi (LLR)	PT3+4	53
	R-C	21 Kozi (15 LLR, 6 LR), 14 Kala (LR), 8 Bahn (1 LLR, 7 LR), 4 Sajd (3LLR, 1 LR)	PT1,3+4	47
	R-D	10 Bors (LR), 8 Sast (LR), 7 Kozi (4 LLR, 3 LR), 6 Bahn (LR), 6 Brod (LR), 5 Sajd (2 LLR, 3 LR), 1 Kala (LR)	PT1-4	43
	R-E	14 Kozi (10 LLR, 4 LR), 7 Bahn (2 LLR, 5 LR), 6 Sajd (5 LLR, 1 LR), 2 Kala (LR)	PT1,3+4	29
	R-F	18 Sats (LR), 1 Kala (LR)	PT1+2	19
	R-G	11 Kozi (8 LLR, 3 LR), 1 Sajd (LLR)	PT3+4	12
	R-H	6 Bors (LR), 5 Sast (LR), 1 Kala (LR)	PT1+2	12
	R-I	8 Kala (LR), 1 Bahn (LR), 1 Bors (LR)	PT1+3	10
	R-J	5 Sast (LR), 3 Brod (LR), 1 Bahn (LR)	PT2+3	9
	R-K	8 Sast (LR)	PT1+2	8
	R-L	6 Sast (LR), 1 Bors (LR)	PT1+2	7
	R-M	5 Kozi (LLR), 1 Sajd (LLR)	PT3+4	6
	R-N	2 Sast (LR), 1 Bors (LR) 1 Brod (LR)	PT1+2	4
	Single MLG			51
	Total			366
L (among LR)	L-A	38 Boro	PT4	38
	Single MLG			245
	Total			283
LL (among LLR)	LL-A	52 Kozi (LLR), 20 Sajd (LLR), 5 Bahn (LLR)	PT3+4	77
	LL-B	6 Boro (LLR)	PT4	6
	Single MLG			0
	Total			83
CombLLR (among LLR)	Comb-B	15 Kozi, 3 Sajd, 1 Bahn (composed of LL-A + R-C)	PT3+4	19
	Comb-C	10 Kozi, 5 Sajd, 2 Bahn (composed of LL-A + R-E)	PT3+4	17
	Comb-D	9 Kozi, 4 Sajd, 2 Bahn (composed of LL-A + R-A)	PT3+4	15
	Comb-E	8 Kozi. 1 Sajd (composed of LL-A + R-G)	PT3+4	9
	Comb-F	4 Kozi, 2 Sajd (composed of LL-A + R-D)	PT3+4	6
	Comb-G	5 Kozi, 1 Sajd (composed of LL-A + R-M)	PT3+4	6
	Comb-H	3 Boro (composed of LL-B + R-B)	PT4	6
	Single MLG			11
	Total			83
Comb LR (among LR)	Comb-A	35 Boro (composed of L-A + R-B)	PT4	35
	Single MLG			248
	Total			283

Figures

Figure 1: Locations of the sampled populations.

Figure 2: Clustering tree based on F_{ST} matrices of frog types from each population for a) L and b) R genomes.

Figure 1

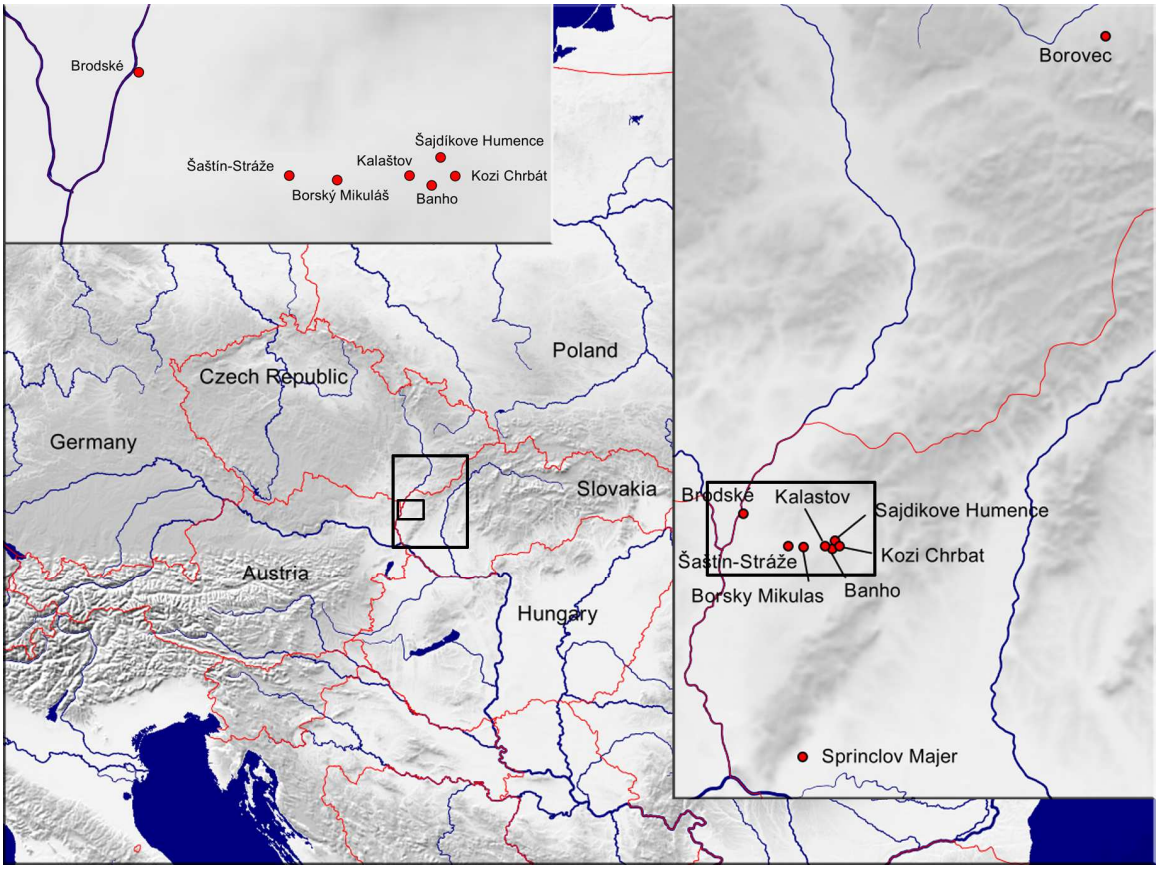
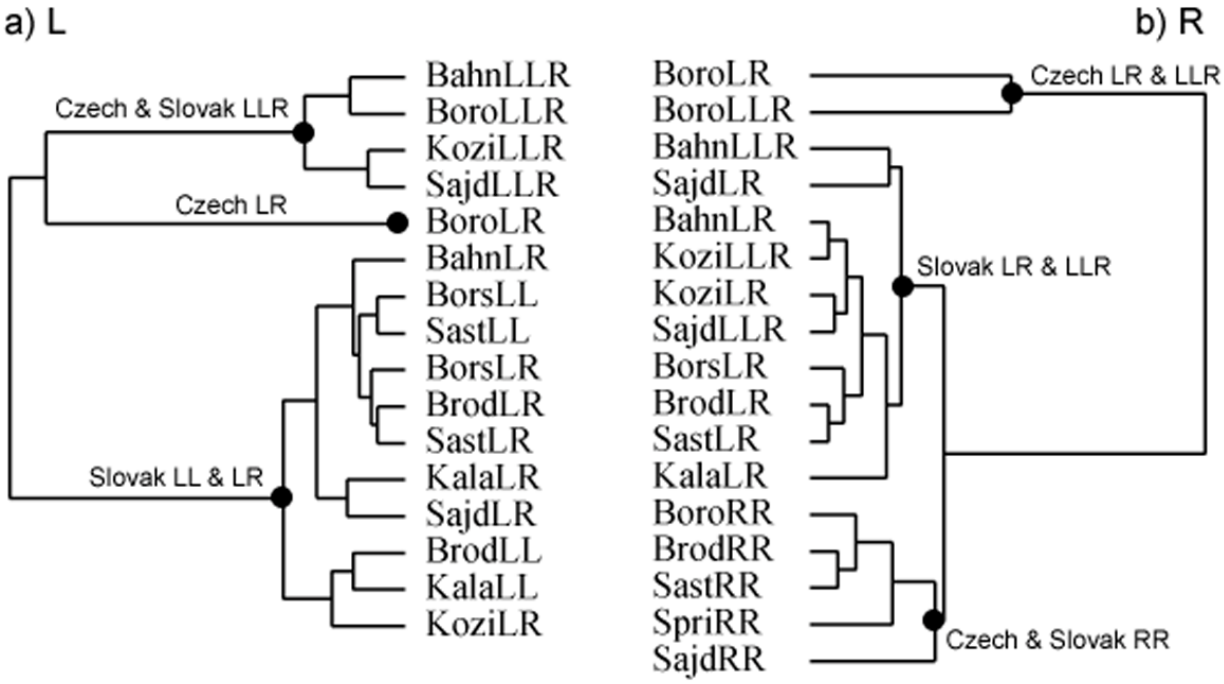

Brodské
Šajdíkove Humence
Kalaštov
Šaštín-Stráže
Kozi Chrbát
Borský Mikuláš
Banho
Poland
Czech Republic
Germany
Slovakia
Austria
Hungary
Borovec
Brodske
Kalastov
Sajdikove Humence
Šaštín-Stráže
Kozi Chrbat
Borsky Mikulas
Banho
Sprinclov Majer

Figure 2


a) L
Czech & Slovak LLR
Czech LR
Slovak LL & LR
BahnLLR
BoroLLR
KoziLLR
SajdLLR
BoroLR
BahnLR
BorsLL
SastLL
BorsLR
BrodLR
SastLR
KalaLR
SajdLR
BrodLL
KalaLL
KoziLR
b) R
Czech LR & LLR
Slovak LR & LLR
Czech & Slovak RR
BoroLR
BoroLLR
BahnLLR
SajdLR
BahnLR
KoziLLR
KoziLR
SajdLLR
BorsLR
BrodLR
SastLR
KalaLR
BoroRR
BrodRR
SastRR
SpriRR
SajdRR

Appendix

A1: Gene diversity corrected for sample size, Nei 1978 (He) for *P. lessonae* genomes and *P. ridibundus* genomes, broken down by locus, genotypes and population. Sample size is given in brackets.

L Genome	All (415)	LL (49)	LLR (83)	LR (283)	RR	BahnLLR (5)	BahnLR (31)	BoroLLR (6)	BoroLR (50)	BorRR	BorsLL (15)	BorsLR (24)	BrodLL (4)	BrodLR (35)	BrodRR	KalaLL (3)	KalaLR (32)	KoziLLR (52)	KoziLR (20)	SajdLLR (20)	SajdLR (12)	SajdRR	SastLL (27)	SastLR (79)	SastRR	SpriRR
CA1b6	0.491	0.495	0.503	0.465	-	0.556	0.516	0.546	0.150	-	0.481	0.489	0.607	0.511	-	0.600	0.315	0.505	0.442	0.513	0.530	-	0.492	0.456	-	-
RlCA1b5	0.196	0.340	0.000	0.218	-	0.000	0.323	0.000	0.000	-	0.370	0.228	0.250	0.161	-	0.600	0.175	0.000	0.479	0.000	0.303	-	0.308	0.243	-	-
Res20	0.770	0.838	0.000	0.841	-	0.000	0.858	0.000	0.322	-	0.777	0.895	0.857	0.728	-	0.867	0.839	0.000	0.368	0.000	0.803	-	0.811	0.781	-	-
RlCA2a34	0.786	0.807	0.503	0.815	-	0.556	0.736	0.546	0.187	-	0.770	0.815	0.750	0.894	-	0.600	0.694	0.505	0.747	0.513	0.439	-	0.785	0.719	-	-
Ga1a23	0.865	0.874	0.503	0.870	-	0.556	0.733	0.546	0.382	-	0.853	0.634	0.964	0.808	-	0.733	0.774	0.505	0.763	0.513	0.758	-	0.864	0.867	-	-
Rrid013A	0.547	0.429	0.000	0.338	-	0.000	0.241	0.000	0.040	-	0.508	0.562	0.250	0.406	-	0.000	0.546	0.000	0.100	0.000	0.167	-	0.416	0.347	-	-
CA1a27	0.704	0.678	0.000	0.688	-	0.000	0.669	0.000	0.376	-	0.756	0.377	0.607	0.548	-	0.800	0.712	0.000	0.468	0.000	0.758	-	0.612	0.462	-	-
RlCA18	0.677	0.658	0.537	0.631	-	0.556	0.641	0.546	0.346	-	0.687	0.688	0.464	0.563	-	0.600	0.534	0.505	0.595	0.513	0.530	-	0.658	0.592	-	-
Mean	**0.629**	**0.640**	**0.256**	**0.608**	-	**0.278**	**0.590**	**0.273**	**0.225**	-	**0.650**	**0.586**	**0.594**	**0.577**	-	**0.600**	**0.574**	**0.252**	**0.495**	**0.256**	**0.536**	-	**0.618**	**0.558**	-	-
Stand. Error	0.076	0.071	0.097	0.086	-	0.105	0.076	0.103	0.054	-	0.061	0.078	0.093	0.083	-	0.093	0.081	0.095	0.076	0.097	0.081	-	0.071	0.077	-	-
R Genome	All (475)	LL	LLR (83)	LR (283)	RR (109)	BahnLLR (5)	BahnLR (31)	BoroLLR (6)	BoroLR (50)	BoroRR (19)	BorsLL	BorsLR (24)	BrodLL	BrodLR (35)	BrodRR (52)	KalaLL	KalaLR (32)	KoziLLR (52)	KoziLR (20)	SajdLLR (20)	SajdLR (12)	SajdRR (2)	SastLL	SastLR (79)	SastRR (26)	SpriRR (10)
CA1b6	0.611	-	0.563	0.533	0.770	0.600	0.443	0.000	0.078	0.431	-	0.540	-	0.612	0.771	-	0.508	0.594	0.542	0.600	0.318	0.667	-	0.659	0.798	0.616
RlCA1b5	0.062	-	0.000	0.007	0.235	0.000	0.000	0.000	0.040	0.273	-	0.000	-	0.000	0.277	-	0.000	0.000	0.000	0.000	0.000	0.000	-	0.000	0.214	0.000
Ga1a19	0.433	-	0.394	0.267	0.730	0.400	0.426	0.000	0.000	0.542	-	0.083	-	0.292	0.767	-	0.508	0.419	0.442	0.426	0.167	0.000	-	0.167	0.610	0.884
Rrid064A	0.422	-	0.516	0.321	0.555	0.400	0.396	0.333	0.000	0.246	-	0.453	-	0.308	0.697	-	0.514	0.594	0.616	0.353	0.318	0.667	-	0.167	0.372	0.532
Re2Caga3	0.802	-	0.787	0.754	0.749	0.800	0.772	0.600	0.078	0.643	-	0.685	-	0.731	0.749	-	0.698	0.790	0.826	0.768	0.712	0.667	-	0.729	0.753	0.811
Res22	0.600	-	0.518	0.535	0.683	0.600	0.514	0.000	0.000	0.495	-	0.518	-	0.504	0.620	-	0.627	0.507	0.479	0.563	0.303	0.667	-	0.493	0.828	0.711
Rrid169A	0.552	-	0.376	0.408	0.694	0.400	0.525	0.000	0.040	0.690	-	0.228	-	0.494	0.737	-	0.542	0.419	0.442	0.353	0.167	0.000	-	0.383	0.514	0.605
Rrid013A	0.079	-	0.000	0.014	0.274	0.000	0.000	0.000	0.000	0.309	-	0.000	-	0.111	0.305	-	0.000	0.000	0.000	0.000	0.000	0.000	-	0.000	0.298	0.000
Rrid059A	0.398	-	0.024	0.305	0.741	0.000	0.185	0.000	0.000	0.383	-	0.562	-	0.461	0.778	-	0.123	0.000	0.000	0.100	0.167	0.667	-	0.461	0.795	0.647
Re1Caga10	0.777	-	0.690	0.705	0.873	0.800	0.680	0.333	0.040	0.762	-	0.554	-	0.657	0.890	-	0.613	0.679	0.700	0.711	0.439	0.833	-	0.576	0.781	0.690
Rrid135A	0.694	-	0.672	0.707	0.639	0.800	0.738	0.000	0.040	0.677	-	0.612	-	0.630	0.629	-	0.464	0.660	0.668	0.679	0.439	0.667	-	0.723	0.655	0.542
Mean	**0.494**	-	**0.413**	**0.414**	**0.631**	**0.436**	**0.425**	**0.115**	**0.029**	**0.496**	-	**0.385**	-	**0.436**	**0.656**	-	**0.418**	**0.424**	**0.429**	**0.414**	**0.275**	**0.439**	-	**0.396**	**0.602**	**0.549**
Stand. Error	0.075	-	0.086	0.079	0.061	0.097	0.081	0.063	0.009	0.055	-	0.077	-	0.070	0.059	-	0.076	0.088	0.090	0.085	0.063	0.106	-	0.083	0.067	0.088

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Genotype-temperature interactions on larval performance shape population structure in hybridogenetic water frogs (*Pelophylax esculentus* complex)

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Abstract.- The evolutionary potential and ecological importance of interspecific hybrids continues to be a controversial issue. Traditionally, hybridization - often associated with polyploidy and clonal reproduction - was considered an important mechanism for speciation in plants, but not in animals. More recently, investigations have shifted to the question: Under which genetic and ecological conditions do hybrid taxa and different ploidies arise and succeed, and when and where do they fail? Finding answers to this question is aggravated by the fact that suitable taxa for such studies are often far apart on the phylogenetic tree. Hence, results are influenced by many confounding variables.

In this study, we reduce this problem by investigating the fitness within a complex of three closely related water frog taxa consisting of the two sexually reproducing parental species *Pelophylax lessonae* (genotype LL) and *P. ridibundus* (RR) plus their interspecific hybrid *P. esculentus* which comes in three ploidy types (LR, LLR, LRR), as well as with sexual and hemiclonal reproduction. Offspring of all five genotypes were produced by artificially crossing adults sampled from populations in Slovakia, Germany and Switzerland. This created genetic variation. They were then raised at two temperature-levels: 18ºC and 24ºC. This created ecological variation. Larval performance under the two temperature regimes was analysed with respect to three fitness-related parameters: survival rate, days to metamorphosis and weight at tail resorption.

Survival rate was significantly higher for offspring of the three hybrid types (LR, LLR, LRR) compared to those of the parental species (LL, RR), at both rearing temperatures. For days to metamorphosis and weight at metamorphosis we found an interaction between offspring type and temperature. In both cases, performance of hybrid and parental offspring did not differ at 24ºC, but at 18ºC hybrids metamorphosed faster and at a lower weight than parentals.

We discuss these results in relation to those from other studies and conclude that under cold conditions hybrids (especially the two triploid types) have higher fitness than both parental species. This genotype x environment interaction could be one reason why all-hybrid populations mainly occur at the cooler northern range of the water frog distribution.

Keywords: Hybridogenesis; clonal reproduction; ploidy; all-hybrid populations; survival; larval development; metamorphosis; temperature; genetic compatibility; *Pelophylax esculentus*

Introduction

The evolutionary potential and ecological importance of interspecific hybrids has been a controversial issue for quite some time. While seen as an important process for speciation by botanists (Grant 1981), zoologists traditionally dismissed hybridization as an evolutionary dead end (Mayr 1963). The diverging views originated from the, on average, higher number of successful hybrids in plants than in animals (reviewed by Grant 1981, Arnold 1997, Mallet 2005). However, within both kingdoms hybridization is very unequally distributed among taxa, and in some animals its rate even exceeds that in plants (Grant and Grant 1992, Ellstrand et al. 1996, Mallet 2005). Hence, analyses of the evolutionary and ecological role of hybridization should extend the specific comparison between plants and animals to the more general question: What traits and environmental conditions separate taxa with successful hybridization from those where it does not occur in the first place (pre-zygotic selection) or leads to unfit offspring (post-zygotic selection)?

Important features that are often linked to hybridization are clonal reproduction and polyploidy, i.e. the existence of three or more complete chromosome sets, rather than two. Recent crossing experiments by Choleva et al. (2012) with spined loaches (Cobitis) suggest that clonality may be "directly triggered by interspecific hybridization and that polyploidy is a consequence, not a cause, of clonality." The probability of establishing a successful, i.e. evolutionary significant, polyploid hybrid lineage is a function of several factors. These include pre-zygotic ones like the rate at which unreduced gametes are produced and the likelihood that they will fuse. They also include post-zygotic ones like the viability and fertility of the resulting offspring and their competitive ability, relative to offspring of the parental species (cf. Vrijenhoek 1989, Soltis and Soltis 1999, Otto and Whitton 2000, Coyne and Orr 2004, Mable 2004). In this paper, we focus on the post-zygotic factors which determine the success of polyploids, once they have been formed.

Whether polyploid zygotes are produced and develop into viable and fertile polyploid offspring depends on genetic compatibility between the genomes of the two species that hybridize ("balance hypothesis"; Moritz et al. 1989). Even when phenotypically viable, allopolyploids (i.e. those arising from hybridization between different species) are often genetically unfit, due to meiotic problems. The fact that approximately two thirds of allopolyploid animals have abandoned recombination between the parental genomes and reproduce clonally testifies to the importance of avoiding meiotic disturbances (reviewed by Vrijenhoek et al. 1989, Beukeboom and Vrijenhoek 1998, Otto and Whitton 2000). Solving the meiotic problem through shifting to clonal reproduction comes at the expense of reduced genetic diversity available for adaptation and the risk of accumulating deleterious alleles through Muller's ratchet (Muller 1964). This is why several authors have considered polyploids and clonal organisms "evolutionary dead ends", at least as far as individual lineages are concerned (e.g. Vrijenhoek 1989, Maynard Smith 1992).

Even when the genetic problems can be overcome, successful establishment of allopolyploid hybrids may be prevented for ecological reasons. As hybrids are usually intermediate in their characteristics and

requirements, they will compete with both parental species that are adapted to and usually superior in different niches along an ecological gradient.

Despite these problems, some clonal and hemiclonal hybrids are ecologically and evolutionary fairly successful (for recent reviews see Arnold 1997, Kearney 2005, Avise 2008, Hörandl 2009, Vrijenhoek and Davis Parker 2009). Ecological explanations for the success of hybrids assume that they can reduce competition by inhabiting different, intermediate or broader niches than their progenitors (Moore 1984, Otto and Whitton 2000, Seehausen 2004). Genetic explanations for the success include (a) occasional incorporation of new nuclear material from the sexual host (Hedges et al. 1992, Spolsky, et al. 1992, Schartl et al. 1995), (b) formation of different clonal lineages through repeated primary hybridization (Soltis and Soltis 1999, Little and Hebert 1997, Janko et al. 2003, Moritz et al. 1989, Ptacek et al. 1994, Stöck et al. 2005), (c) spontaneous heterosis (“hybrid vigor”; Lippmann and Zamir 2007) and (d) “transgressive segregation”, i.e. the production of extreme phenotypes that exceed the combined range of trait values of both parental lines (Rieseberg et al. 1999, Stelke and Seehausen 2009). Mechanisms (c) and (d) are based on the fact that the combination of different parental genomes and/or the addition of extra genomes can lead to increased levels of somatic heterozygosity in hybrids. This may explain why allopolyploids and other hybrids seem to be better adapted than the parental species to invade and establish themselves in novel, perturbed and extreme habitats and, as a result, are found in high proportions at the geographical periphery of species ranges and in harsh environments at high latitudes and altitudes (Otto and Whitton 2000, Mable 2004, Seehausen 2004).

Most of the above conclusions stem from inter-specific comparisons, often between taxa that are far apart on the phylogenetic tree. Since such comparisons are strongly affected by several confounding variables, comparisons within species or complexes of very closely related organisms are to be preferred for investigating how genetics and ecological competition affect the success of clonally reproducing hybrids with different ploidies, relative to their sexual parental species. However with a few exceptions (Cullum 1997, Alves et al. 2001, Pala and Coelho 2005, Stöck et al. 2005,

2010), this has rarely been attempted for vertebrates, because the necessary intra-specific variation is lacking.

The *Pelophylax* study system

An excellent system for such a comparison is provided by the Edible Frog *Pelophylax esculentus* (called *Rana esculenta* until Frost et al. 2006). Originally derived from matings between the pool frog *P. lessonae* (phenotype L, genotype LL) and the marsh frog *P. ridibundus* (R, RR), *P. esculentus* (E, LR) combines hybrid origin with hemiclonal reproduction and – in some populations – with polyploidy (LLR, LRR). This allows intra-specific comparisons between hybrids of different ploidies and intra-complex comparisons between hybrids and their two closely related parental species.

Prior to meiosis, *P. esculentus* eliminates one of the parental genomes, duplicates the remaining genome and transmits it clonally to eggs and sperm cells (“hybridogenesis”; Schultz 1969, Tunner 1974, Uzzell et al. 1980, Graf and Polls Pelaz 1989). As a result of this gametogenesis mechanism, hybrid x hybrid matings lead to larvae of the parental type whose genome is clonally transmitted, i.e. to RR in case of R genome transmission and to LL in case of L genome transmission (“hybridolysis”, Günther and Plötner 1988, Plötner 2005). However, these parental types of hybrid origin usually do not survive to metamorphosis, because recessive lethal alleles have accumulated on the clonally transmitted genome through the Muller’s ratchet mechanism (Berger 1977, Graf and Müller 1979, Uzzell et al. 1980, Vorburger 2001a). Hence, *P. esculentus* is a sexual parasite that must live in sympatry and backcross with the parental species (sexual host) whose genome it eliminates (Schmidt 1993, Joly 2001).

Depending on the specific genetic interactions between the hybrid and the parental species, three major breeding systems can be distinguished: the LE-, RE- and EE-system (Graf and Polls Pelaz 1989, Günther 1990, Plötner 2005). The most widespread and best investigated one is the LE-system, where the hybrids exclude the L genome and breed with *P. lessonae* to re-establish hybridity at each generation (Table 1a). The mirror system to this is the RE-system where hybrids exclude the R genome and backcross with *P. ridibundus* to perpetuate themselves (Table 1b). The all-hybrid EE-system

(Table 1c), with no parental sexual host to mate with, seems to defy the rules and mechanisms outlined above. Yet, such populations have been found in several parts of Europe, and they remain stable over many years (Christiansen et al. 2010). The key to their success is the existence of and mating between diploid (LR) and triploid hybrids (LLR, LRR). In EE populations, the triploids replace the parental species as sexual hosts by providing the haploid L (LLR) or R gametes (LRR) that in LE and RE populations are produced by LL and RR individuals, respectively.

When genotypes, sex ratios and gamete production patterns are included, several variations of these three basic breeding systems are found in Europe. As a result, the composition of a population (defined by the genotypes of the occurring animals) does not always enable us to deduce the breeding system (defined by genetic interactions) it belongs to. In this paper we therefore speak of population types, rather than breeding systems.

In populations where diploid hybrids occur in sympatry with parental species (in this study represented by an $LE_{2n}R$-population from Slovakia and an LE_{2n}-population from Switzerland) both sexes of all genotypes usually produce haploid gametes. In all-hybrid populations (in this study represented by an $E_{2n}E_{3n}$-population from Germany), there is some variation in gamete types of diploids hybrids, but the most frequent pattern is the one shown in Table 1c (based on Jakob 2007, Christiansen 2009). Considering this pattern of gamete production and all possible mating combinations, we not only expect LR, LLR and LRR hybrids from hybrid x hybrid matings, but also offspring of both parental species, *P. lessonae* (LL) and *P. ridibundus* (RR) (Table 1c). In all-hybrid $E_{2n}E_{3n}$-populations, these do actually occur during larval stages, but no longer exist among adults (Arioli 2007).

In this study, we investigate genetic and ecological factors that might affect the composition of water frog populations via fitness differences between offspring of the two parental species and those of diploid and triploid hybrids. Larvae were produced by artificially crossing adults from different geographical regions, different breeding systems and of different ploidies. This introduced genetic variation. They were raised under two temperatures, which introduced ecological variation. Fitness was measured by three variables that in amphibians are known to represent good correlates: tadpole survival, time

to metamorphosis and weight at metamorphosis. Results from the experiment are used to discuss why some water frog populations are mixed, with parental species and hybrids living in sympatry, whereas others consist of hybrids only.

Materials and methods

Source populations

The adults used for crossing originated from three European countries (Figure 1). In Germany, frogs were caught from the village pond of Schönermark (52°54'08"N, 12°19'16"E), near Kyritz; in Switzerla nd in a pond near Hellberg (47°17'36"N, 8°48'29"E), Canton of Zurich; and in S lovakia from four ponds, all located within 10 km of Šaštín-Stráže (48°37'55 "N, 17°08'40"E). The Kyritz population is an all-hybrid $E_{2n}E_{3n}$-population with diploid LR and triploid LLR and LRR. The Šaštín-Stráže population consists of diploid LR and both parental species, LL and RR ($LE_{2n}R$-population). The Hellberg pond represents an LE_{2n} population where the diploid hybrids occur in sympatry with only one parental species (LL). In all three areas, the mentioned genotypes occur in both sexes, but from the Hellberg pond only LL females were included in the crossing design (see below).

Frogs were caught at night by hand while dazzling them with a strong flashlight. Sex was determined on the spot based on the presence (males) or absence (females) of thumb pads and vocal sacs. For preliminary genotype determination we used the shape of the Callus internus and produced blood smears on slides for subsequent measurement of erythrocyte length and width. This allows identification of ploidy since triploids have larger cells than diploids (Polls-Pelaz and Graf 1988, Jakob 2007), but it does not allow unambiguous distinction between individuals of the same ploidy, i.e. between LL, RR and LR or between LLR and LRR. Therefore, all frogs were toe clipped for later genotype identification through microsatellite analysis. For transport to Zurich, selected frogs were individually marked with transponders (ID-162, AEG), separated by sex and assumed genotype, stored in cloth bags filled with rubber sponges, and showered daily with fresh water. All frogs survived the journey.

Microsatellite analysis

Precise genotype identification of both the parental frogs and the offspring resulting from the crosses was achieved through microsatellite analysis using a piece of the tailfin (tadpoles) and a fingertip (adults, metamorphs) respectively as the source material. DNA extraction and purification were performed using a Biosprint 96 DNA Blood Kit (Qiagen) in combination with the Biosprint 96 workstation following the supplier's protocol. The purified DNA was subjected to PCR runs with four primer mixes involving a total of 18 microsatellites primer pairs (Table 2). Details on PCR protocols are given by Christiansen (2009) and Christiansen and Reyer (2009, 2011). PCR products were run for fragment length analysis on an ABI 3730 Avant capillary sequencer with internal size standard (GeneScan-500 LIZ), and the alleles were scored with the GeneMapper software v3.7 (Applied Biosystems). Loci Res20, RlCA1a27 and RlCA18 were species-specific for *P. lessonae*, and Res22, Rrid169A and Re2CAGA3 were species-specific for *P. ridibundus*. The other 12 loci amplified in both *P. lessonae* and *P. ridibundus* genomes (Christiansen 2005, 2009, Arioli et al. 2010). Moreover, loci CA1b6, RlCA1b5, Ga1a19redesigned and Res16 showed a dosage effect which was used to detect triploidy by comparing the relative height of the peaks (Christiansen 2005). Knowing the genotypes of the parents and their offspring we could infer the genotype and ploidy of the gametes they originated from. This also allowed us to check for possible aneuploidy of the offspring, which did not occur.

Crossing procedure

Crosses were performed through artificial fertilization, following the protocol of Berger et al. (1994) with the following modifications. To stimulate ovulation, females were injected with LHRH fish hormone (Bachem H-7525, 2 mg in 100 ml Holtfreter's solution). After about 24 hours, when eggs were ready for being stripped off, males were euthanized in a buffered (pH 7) MS-222 solution (Sigma A-5040) at 1mg/l. Their testes were removed and a piece crushed in a Petri dish with aged tap water. Eggs were stripped into this sperm suspension, where they remained for about 2-3 minutes. After this period, the suspension was rinsed into a new Petri dish to which eggs of another female were added.

This allowed using the same sperm solution to fertilize eggs from different females. After fertilization, eggs were covered with aged tap water and checked for fertilization success. This can be easily identified since fertilized eggs rotate their black animal hemisphere to the top within 30-60 min.

The next day, all eggs were transferred to containers with 1 litre of water with a water-air interface of 600 cm^2 (20x30 cm). After two days unfertilized eggs and/or aborted embryos were carefully removed every two days to avoid bacterial and fungal development. After 15 days embryos had reached the free swimming tadpole stage (Gosner stage 23-25; Gosner 1960).

Experimental design

The crossing procedure described above allows fertilization of eggs from different females with sperm from the same male and, conversely, fertilization of eggs from the same female with sperm from different males. In this way, one can produce half-sib offspring cohorts within and between populations. In our study, we crossed males and females of different origin both within and between genotypes and locations, respectively. Originally, we had planned a fully crossed design with three replicates for all combinations. However, due to insufficient egg numbers in some females and/or failed fertilization through sperm of some males only the 57 crosses shown in Table 3 could be performed. Although there are some gaps, the design is complete in the sense that all adult genotype x location combinations are represented. Thus, the conditions for testing how offspring performance is affected by type and origin of the parents are fulfilled. All crosses involved at least one hybrid parent. We did not perform crosses between parental males and females. Given the above mentioned shortage of eggs and/or failed fertilization through male sperm, inclusion of such crosses would inevitably have led to gaps and reduced the number of replicates for crosses involving one or two hybrids. And offspring from these crosses are more relevant for our questions than offspring resulting from crosses between the two parental species.

Rearing of tadpoles

After reaching the free swimming stage, groups of five healthy looking tadpoles from the same cross were transferred to 5 litres tubs containing 3.5 litres of aged tap water. Additional tadpoles (usually 25 per cross) were used for determining their genotypes and the gamete types produced by the crossed males and females but not further considered in this study. The rearing tubs were placed on four layers of shelves in two climate chambers, one set to 19ºC and the other to 25ºC (+/- 1ºC). Due to the cooling effect of evaporation this resulted in water temperatures of 18º and 24ºC, respectively. The climate chambers (SR Kältetechnik and Partner) were illuminated by lamps (tulux, 18W/230V/50Hz) from 6 am to 9 pm (15L:9D regime). Initial arrangement of the boxes on the shelves and weekly changes of their locations were made using the randomizing function in Excel. Since performing all crosses took four days (2.6.2009-5.6.2009) and hence tadpoles differed in age, their transfer to the climate chamber was staggered correspondingly, so that all tadpoles entered the temperature treatment at the same age.

Tadpoles were fed once a week with a powder mix consisting of 4 parts rabbit food (plant material) and 1 part Spirulina tabs (vitamins and algae). Food was provided using a custom-made spoon containing a mean of 0.0114g (+/- 0.0016 g). Feeding was adjusted to the number of tadpoles still alive in a tub by adding one spoon of food per tadpole. Following some mortality after six weeks (11 dead tadpoles out of 335 at 18ºC and 9 dead ones out of 320 at 24ºC), the feeding schedule was increased to two times a week. Water was changed every three days, with the transfer date as the reference point. We always used aged tap water that had been equilibrated to the room temperature of the respective treatments.

Statistical analyses

We recorded three parameters that are frequently used for describing tadpole performance: days to metamorphosis, survival to metamorphosis, and weight at metamorphosis. Metamorphosis was defined as emergence of at least one forelimb (stage 42; Gosner 1960). The number of days from fertilization to this stage was used as a measure for days to metamorphosis, and the number of

tadpoles reaching this stage was used to calculate survival to metamorphosis. Tadpoles that had survived but not yet metamorphosed when the experiment was terminated 169 days after fertilization were considered non-survivors. The best estimate for body size at metamorphosis is the weight at tail resorption (Travis 1980, 1984). Therefore, metamorphs were held separately in Petri dishes containing humidified cotton until tail resorption was complete and then weighed to the nearest 0.1 mg.

Prior to analysis, the three fitness parameters were tested for their distribution using the Kolmogorov-Smirnov one sample test with Lilliefors modification. Since variables were not normally distributed, and in order to increase additivity of effects and equality of variance (Snedecor and Cochran 1980) days to and weight at metamorphosis were logarithmically transformed and survival rate was transformed by the arcsine of the square-root.

General linear models (GLM) were used to relate these three parameters to the following four factors: two classes of experimentally manipulated temperatures [18º and 24ºC], two parent origins [same population (S), different populations (D)], two parental combinations [both hybrids (H-H), one hybrid and one parental species (H-P)] and five offspring-genotypes [LL, LLR, LR, LRR, RR], respectively two categories, hybrids [LLR, LR, LRR] and parentals [LL, RR]. Two-way interactions between the four factors were also included in the model. Factors with significant effects were subsequently subjected to pairwise comparisons using Scheffe's test. All tests were performed using Systat 11.0 (Systat Software Inc.).

A few crosses resulted in mixed offspring genotypes (e.g. LR and LLR or LL and LLL) because some adults produced two gamete types (cf. Table 3). These mixed cohorts were included in the GLM with two offspring categories, but not in the one with the five genotypes. Our crosses also generated a few autotriploids (LLL and RRR) that occurred in mixed cohorts with mostly diploid larvae of the parental species (LL and RR). Although there is some debate whether performance of autotriploids differs from that of autodiploids (e.g. Stebbins 1985, Parisod et al. 2010) pooling of LLL and RRR with LL and RR could not bias our results because there were only three autopolyploid tadpoles in our rearing experiment: one RRR in cross female 015-68 x male 014-48 at 18ºC, one LLL in cross 014-62 x 015-50 at 18ºC and

one LLL in cross 014-21 x 014-56 at 24ºC (see Table 3). None of those three individuals reached metamorphosis before the end of the experiment. So they were not included in the analyses of days to and weight at metamorphosis and must have had a negligible impact on survival values.

Results

Gamete and offspring types

The artificial crosses produced five types of pure offspring cohorts (LL, LLR, LR, LRR and RR) and four types of mixed cohorts (LR/LLR, LR/LRR, LL/LLL and RR/RRR). Combined with the known genotypes of the crossed adults, this allows identification of the gametes produced by males and females (Table 3). In males, all individuals produced exclusively haploid gametes, independent of their genotype and origin. Female gamete production was more diverse and varied with both genotype and locality. In Kyritz, all triploid female hybrids produced the expected haploid gametes, namely L eggs in LLR and R eggs in LRR individuals; but LLR females also produced a small number of diploid LL eggs (average of 13.5%). Among the diploid female hybrids, those from Kyritz produced diploid LR eggs, while those from Šaštín-Stráže produced haploid R eggs. All females of the two parental species produced the expected haploid eggs, but one RR female from Šaštín-Stráže produced also a few diploid RR eggs (average of 1.5%).

Tadpoles performance

Table 4 shows the results from the three GLM analyses relating offspring survival, days to metamorphosis and weight at metamorphosis to the four experimentally manipulated factors and their two-way interactions. The most consistent significant effects on tadpole performance were exerted by temperature and offspring genotype. At 24ºC, survival was significantly higher, time to metamorphosis was shorter and weight at metamorphosis was lower than at 18ºC (Table 4, Figures 2b-c). Survival was lowest for offspring with the two parental genotypes (LL, RR) and highest for those of the three pure hybrid cohorts (LR, LLR, LRR) (Figure 3a). At 18ºC, the pattern for days to and weight at metamorphosis was basically a mirror image of the survival pattern:

highest values for LL and RR and lowest values for LR, LLR and LRR (Figures 3b, c). Pairwise comparisons revealed no significant differences between the two parental species and the three hybrid types, respectively, for any of the three performance measures. When results from cohorts of the three pure hybrid types and the two parental species are pooled into two categories (right sides of Figures 3a-c), differences between the hybrids and parental species are significant for survival. For days to, and weight at, metamorphosis they are significant at 18ºC, but not at 24ºC; hence the offspring type x temperature interactions for these two variables (Table 4). For days to metamorphosis, pairwise tests of this interaction showed significant differences between developmental rate at 18º and 24ºC for the three diploid genotypes (LL, LR, RR), but not for the two triploid ones (LLR, LRR).

As tadpoles that had not metamorphosed until the end of the experiment (day 169) were considered non-survivors, survival values are potentially confounded by long development times. This is supported by the significant relationship between survival and the number of tadpoles remaining at the end of the experiment (Table 4). However, the above mentioned effects of temperature and offspring type on survival emerged, even though tadpole number was included in the analysis. Survival, but not time to and weight at metamorphosis, was also influenced by the combination of the parents. Survival of offspring from crosses between hybrid males and females was significantly lower than survival of offspring from crosses where only one parent was a hybrid and the other belonged to a parental species (Figure 2a). Crosses within and between populations (population type) produced no significant differences for any of the three performance variables, nor did the two-way interactions, with the exception of the above mentioned offspring type x temperature interaction.

Discussion

The results of this study show that development of water frog tadpoles is affected by both ecological factors (here represented by temperature) and genetics (here represented by genotypes of parents and offspring). Both factors influenced all three fitness parameters: survival rate, days to metamorphosis and weight at metamorphosis. Overall, hybrids performed

better than the parental species. It seems plausible to assume that this “hybrid vigor” results from spontaneous heterosis that is due to genetic mechanisms (reviewed by Lippmann and Zamir 2007), like the suppression of deleterious alleles in one parental genome through dominant alleles in the other (dominance hypothesis), a combination of alleles that are particularly advantageous in the heterozygous state (overdominance hypothesis) and/or modification of genes by those at other loci (epistasis). However, evidence for heterosis in clonal and hemiclonal hybrids is mixed. Support for spontaneous heterosis comes from crossing experiments of Hotz et al. (1999) who found better survival, higher growth rate, and shorter time to metamorphosis in offspring of F-1 *P. esculentus* than in those of the two parental species. However, other studies on the same system did not detect heterosis effects with respect to growth, development, oxygen requirement, heat resistance and parasite infection (Plenet et al. 2000, 2005, 2009, Livinchuk et al. 2007, Planade et al. 2009) nor was spontaneous heterosis found in newly synthesized strains of the unisexual fish *Poeciliopsis* (Whetherington et al. 1987). Thus, most studies seem to indicate that heterosis alone is usually not sufficient to explain the (hemi)clonal hybrids’ ecological success. It may, however, operate in conjunction with other mechanisms, such as habitat segregation and/or selection of the fittest clones from a spectrum of genotypes that arose via multiple hybrid events (Hotz et al. 1999, Plenet et al. 2005, Planade et al. 2009). Because of this synergy, heterosis effects will be modified by the environment (Lippmann and Zamir 2007). Hence, they may show up under certain ecological conditions and for some traits, but not in other circumstances.

Therefore, we below discuss the specific results for the three fitness parameters one by one in relation to temperature and genotype. At the end we outline potential consequences for population composition.

Survival rate

Survival rate was much higher at 24ºC than at 18ºC (Figure 2b) and higher in hybrid tadpoles than in those of the parental type, but not different between genotypes within these two offspring categories, i.e. not between LL and RR (including two LLL and one RRR), respectively LLR, LR and LR. The absolute

survival values may, to some extent, have been confounded by long development times, because tadpoles that had survived but not yet metamorphosed when the experiment was terminated 169 days after fertilization were considered non-survivors. However, the risk for such a bias is probably negligible, because our experimental period was much longer than usual development times (e.g. Semlitsch et al. 1997) and the remaining tadpoles will have died anyway. Moreover, to avoid this potential bias, we included the number of tadpoles surviving at the end of the experiment as a covariate into the GLM for survival and, yet, the temperature and genotype effects emerged. Thus, survival differences between temperatures and offspring types are real. Better larval survival at high than at low temperature has been reported for water frogs before (Orizaola and Laurila 2009); but a direct effect of low temperature on mortality does not seem to be widespread in experimental studies of amphibian development. Under natural conditions, however, there often will be an indirect effect via prolonged time to metamorphosis and, hence, extended exposure to aquatic predators and/or risk of pond desiccation (see below).

Results of Negovetic et al. (2001) indicate that LL may be better adapted to warm and LR to cold temperatures. Under lab conditions tadpole survival for the parental species *P. lessonae* (LL) was better at 24ºC, whereas that of diploid hybrid *P. esculentus* (LR) was better at 18ºC. These results were corroborated by the distribution in natural ponds: the proportion of hybrids increased with decreasing water temperature. The authors suggest that this thermal niche differentiation may help parentals and hybrids to coexist, despite of many genetic, ecological and morphological similarities. In our study, we found no offspring type x temperature interaction on survival (Table 4), indicating that temperature affected survival of all genotypes equally. The difference between the results from the two studies may partly be related to differences in larval periods which were much longer in our study. Maybe increasing general mortality late in the larval period has obliterated species differences that may have existed earlier.

The reasons for the overall lower survival of parental offspring types in our study also remain unclear. The usual explanation assumes that high parental type mortality results from the fusion of two clonal hybrid genomes

(cf. Table 1) with the same fixed recessive deleterious mutations (Muller 1964; Vorburger 2001a). In our experiment, however, this could be true for only one out of the 26 cohorts with offspring of the parental types (21 RR, 5 LL; see Table 3) and, thus, cannot explain the high mortality of the parental types. In all other cases, LL and RR tadpoles originated from crossings between males and females from far apart populations with different hemiclones (e.g. female 015-72 x male 014-05) and/or from pairs where one or both parents had recombined the genome prior to gametogenesis (e.g. female 016-49 x male 014-56). This introduces heterozygosity into LL and RR tadpoles through the combination of either different hemiclones or one clonal and one sexual genome. As shown by Vorburger (2001a,b) and Guex et al. (2002), such heterozygosity is sufficient to overcome the effect of deleterious mutations on the clonally transmitted genomes. Even in the one intra-population cross from Šaštín-Stráže where two clonal genomes were combined (female 015-72 x male 015-06 in Table 3) these genomes must not necessarily have been identical with respect to their deleterious mutations. Given that in this population LR hybrids live in sympatry with both parental species primary hybridization, as well as backcrossing between hybrids and parental species, is likely to happen fairly regularly (chapter 3 of this thesis). Backcrossing will lead to recombination of originally clonal R genomes from LR hybrids once they have arrived in sexual RR individuals, whereas repeated primary hybridization will result in different and relatively young hemiclones which are unlikely to have already accumulated many deleterious mutations on the same loci.

Yet, low genetic diversity and/or genome incompatibility may have played a role in our study. This is indicated by the fact that offspring survival was lower in crosses where both parents were hybrids (H-H) than in those where one parent was from a parental species (H-P).

Days to metamorphosis

The faster larval development at high compared to low temperatures found in this study is typical for amphibian species. The pattern has repeatedly been demonstrated in experiments like ours, where tadpoles were raised under different temperature regimes (e.g. Alvarez and Nicieza 2002, Walsh et al.

2008). It also emerges from several studies where time to metamorphosis was found to decrease when pond temperature increased as a result of experimentally lowered water levels (e.g. Loman 1999) or decreasing canopy cover (e.g. Skelly et al. 2002, Hocking and Semlitsch 2008, Van Buskirk 2011).

In contrast to survival, the effect of temperature on days to metamorphosis varied between offspring genotypes, as indicated by the significant genotype x temperature treatment (Table 4). Overall, the hybrids develop faster than the parental species at 18ºC, whereas at 24ºC the two categories do not differ (Figure 3b, right). Pairwise tests on this interaction revealed that the difference between the two temperatures is more pronounced for the diploid offspring forms (LL, RR and LR) than for the triploid ones (LLR, LRR). This suggests that decreasing temperatures affect the development controlling mechanisms in triploids less than in diploids. As a result, triploids may be better adapted to develop under cold conditions than diploids, a pattern that has been found in numerous species, including others frogs (Dufresne and Hebert 1998, Lencioni 2004, Otto et al. 2007). It is also in line with the high proportion of polyploid plants and animals found under the harsh environmental conditions at high latitudes and altitudes and at the geographical periphery of species ranges. The explanation for the higher temperature tolerance seems to lie in the additional genome and the resulting increased levels of somatic heterozygosity which, in turn, leads to changes in many morphological and physiological traits, including larger cell and body size and more enzyme varieties (Otto and Whitton 2000, Mable 2004).

Weight at metamorphosis

The factors that had a significant influence on the weight at metamorphosis were the same as the ones affecting days to metamorphosis: Temperature, offspring genotype and their interaction (Table 4). This is not surprising because – all other things being equal - development time directly affects time for feeding and, hence, growth. As a result, we can expect a positive correlation between time to and weight at metamorphosis. This has been found in numerous studies on amphibian larval development (e.g. Semlitsch 1993, Tejedo et al. 2010) including this study. The overall lower weight at

24ºC than at 18ºC (Figure 2d) can be explained by the shorter development time under warm conditions (Figure 2c), and the weight differences between hybrids and parental species are also related to their respective development times (Figures 3b, c): faster hybrid tadpole development at 18ºC leads to lower weight at metamorphosis, whereas similar developmental rate at 24ºC does not result in weight difference between the two groups Interestingly, at 24ºC (but not at 18ºC) LRR and RR offspring tended to be heavier than LL, LR and LLR. These two heavier groups have an excess of R over L genomes (ratios > 0.67), whereas the three lighter groups have not (ratios < 0.5). Increasing size with increasing R:L genome ratios has also been found among hybrid larvae (LRR, LR, LLR) in natural ponds (Jakob 2007). The differences are likely to reflect a weight effect of the *P. ridibundus* genome, i.e. the genome of the largest species in the water frog complex. According to our present results, the extent of this effect may vary with temperature.

Consequences for population structure and dynamics

Larval anurans exhibit high levels of phenotypic plasticity in life history traits; and survival rates, time to and age at metamorphosis vary markedly among species and with the specific combination of various abiotic and biotic factors (Stahlberg et al. 2001, Van Buskirk and Arioli 2005, Lindgren and Laurila 2009, Van Buskirk 2009, 2011). The resulting multitude of species x environment interactions makes identification of the most important determinants of fitness difficult. However, genotype and temperature are definitely very important factors. They have repeatedly been shown to affect larval performance and this will influence the structure and dynamics of populations directly and indirectly (Hellriegel 2000, Hellriegel and Reyer 2000).

Direct effects arise from differences in developmental rates at the two temperatures. Given that at 18ºC (but not at 24ºC) hybrid larvae metamorphose sooner than those of the parental species (Figure 3b) this will improve their survival under cold conditions in two ways. First, they are exposed to aquatic predators (the major cause of tadpole mortality) for shorter times than larvae of the parental species. Second, after entering their terrestrial habitat, early metamorphosing individuals survive better (Altwegg

and Reyer 2003). Such selective advantage of hybrids under cold conditions has also been shown in other studies on larval and adult *Pelophylax* (Negovetic et al. 2001, Anholt et al. 2003).

Indirect effects on the structure and dynamics of populations arise from reinforcement of the temperature-related survival differences via the mating pattern and genetic effects. Given that mating between LR, LLR and LRR seems to be random (Günther and Plötner 1989, Som and Reyer 2006, B. Rondinelli unpubl. data) an increasing number of hybrids will result in a higher proportion of matings between them. About half of these hybrid-hybrid matings (H-H) result in the parental genotypes LL and RR (Table 1), and offspring from H-H combinations survive worse than those from H-P combinations where one partner is from a parental species (Figure 2a). As a result of these direct and indirect effects, cold temperatures will put *P. lessonae* and *P. ridibundus* at a selective disadvantage, compared to *P. esculentus* so that the parental species will gradually be diluted from mixed parental-hybrid populations, and all-hybrid populations will emerge and persist.

Thus, our finding that under cold conditions *P. esculentus* hybrids in general and triploids in particular are at a selective advantage compared to the parental species *P. lessonae* and *P. ridibundus* offers a new explanation for the observed geographic distribution of different breeding system. The predominance of all-hybrid populations in the cooler northern range of the water frog distribution - e.g. in Sweden, Denmark, Northern Germany and Northern Poland (Plötner 2005) – seems at least in part a result of direct and indirect temperature effects. However, the reasons why many such all-hybrid populations differ markedly in the relative numbers of male and female LR, LLR and LRR is not yet fully understood; but differences in gamete production patterns combined with several abiotic and biotic environmental factors offer the most likely explanation (Christiansen 2009, Christiansen et al. 2010, Jakob et al. 2010, Christiansen and Reyer 2011).

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Tables

Table 1: Offspring types (within bold frame) expected from typical gamete types (in italics) and mating combinations that are possible in a) LE-systems, b) RE-systems and c) an EE-systems consisting of diploid LR and two types of triploids, LLR and LRR. Offspring types in grey fields do not occur among adults, although they are initially produced. The brackets in b) indicate that in most RE-systems female hybrids and the resulting gamete and offspring types do not occur.

a) LE-system	**Males**	LL	LR
Females	***Gametes***	*L*	*R*
LL	*L*	LL	LR
LR	*R*	LR	RR

b) RE-system	**Males**	RR	LR
Females	***Gametes***	*R*	*L*
RR	*R*	RR	LR
(LR)	(*L*)	(LR)	(LL)

c) EE-system	**Males**	LLR		LR		LRR	
Females	***Gametes***	*L*		*R*		*R*	
LLR	*L*	LL		LR		LR	
LR	*LR* *R*	LLR	LR	LRR	RR	LRR	RR
LRR	*R*	LR		RR		RR	

Table 2: Primer mixes and primer pairs used, plus references to published sequences

Primer mix	Primer pairs	Reference for sequences
PM1-A	Ca1b6	Arioli, Jakob & Reyer (2010)
	RlCA1b5	Garner *et al.* (2000)
	Ga1a19redesigned	Arioli, Jakob & Reyer (2010)
	Rrid064A	Christiansen & Reyer (2009)
	RlCA5	Garner *et al.* (2000)
PM2-A	Res22	Zeisset, Rowe & Beebee (2000)
	ReGa1a23	Christiansen & Reyer (2009)
	Rrid169A	Christiansen & Reyer (2009)
	Rrid013A	Hotz *et al.* (2001)
	Rrid059Aredesigned	Christiansen & Reyer (2009)
PM1-B	Res16	Zeisset, Rowe & Beebee (2000)
	Res20	Zeisset, Rowe & Beebee (2000)
	RlCA2a34	Christiansen & Reyer (2009)
	Re2CAGA3	Arioli, Jakob & Reyer (2010)
PM2-B	Re1CAGA10	Arioli, Jakob & Reyer (2010)
	RlCA1a27	Christiansen & Reyer (2009)
	RlCA18	Garner *et al.* (2000)
	Rrid135A	Christiansen & Reyer (2009)

Table 3: Design for artificial crosses between females (vertical) from three populations and males (horizontal) from two populations. Bold letters indicate the genotypes of the individuals, italic letters the gametes they produced. The background color shows whether crossings were between females and males from the same (grey) or from different populations (white). For those crosses that produced fertilized eggs, the genotypes of the resulting offspring are shown in the main body of the table. In cases where a female x male combination resulted in offspring cohorts with two different genotypes, the more frequent one is listed first, but only genotypes printed in bold were included in the rearing experiment. All crosses involved at least one hybrid. For further details on the design see Material and Methods.

Population				Kyritz (DE)								Sastin-Strace (SK)							
	Indiv. numb.	Genotype	Gametes	014-05 **LR** *R*	014-48 **LR** *R*	014-59 **LLR** *L*	014-56 **LLR** *L*	014-55 **LLR** *L*	014-49 **LRR** *R*	014-11 **LRR** *R*	014-58 **LRR** *R*	015-06 **LR** *R*	015-03 **LR** *R*	015-04 **LR** *R*	015-50 **LL** *L*	015-47 **LL** *L*	016-23 **RR** *R*	016-21 **RR** *R*	016-22 **RR** *R*
Kyritz (DE)	014-25	**LR**	*LR*	**LRR**		**LLR**			**LRR**			**LRR**			**LLR**		**LRR**		
	014-63	**LR**	*LR*					**LLR**			**LRR**			**LRR**				**LRR**	
	014-62	**LLR**	*L+LL*	**LR/LLR**		**LL**/(LLL)			**LR/LLR**			**LR/LLR**			**LL/LLL**		**LR**/(LLR)		
	014-21	**LLR**	*L+LL*				**LL/LLL**			**LR**/(LLR)			**LR/LLR**						
	014-24	**LRR**	*R*	**RR**		**LR**			**RR**			**RR**			**LR**		**RR**		
	014-67	**LRR**	*R*		**RR**		**LR**			**RR**									
	014-26	**LRR**	*R*					**LR**						**RR**		**LR**		**RR**	
Sastin-Strace (SK)	015-72	**LR**	*R*	**RR**		**LR**			**RR**			**RR**			**LR**		**RR**		
	015-73	**LR**	*R*		**RR**		**LR**			**RR**									**RR**
	016-49	**LL**	*L*		**LR**		**LL**				**LR**			**LR**					
	015-64	**LL**	*L*																
	015-68	**RR**	*R+RR*	**RR**/(RRR)	**RR/RRR**		**LR/LRR**		**RR**/(RRR)			**RR**/(RRR)							
	015-71	**RR**	*R*								**RR**			**RR**					
Hellberg (CH)	017-44	**LL**	*L*	**LR**		**LL**			**LR**			**LR**							
	017-48	**LL**	*L*																

Table 4: Results from six GLM analyses, two each for the three dependent variables survival, days to metamorphosis and weight at metamorphosis. Values in front of the / refer to analyses with five offspring genotypes (LL, LLR, LR, LRR and RR); values behind the / are from analyses with two offspring categories: hybrids (LLR, LR and LRR pooled) and parental species (LL and RR pooled plus two LLL and one RRR in the survival data set). Significant results are shown in bold. Three-way interactions and one two-way interaction of sources (offspring type x parental combination) could not be included in the analyses because of missing values.

	Survival			Days to metamorphosis			Weight at metamorphosis		
Source	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Temperature	1/1	12·70/30·20	**0·001/0·000**	1/1	81·04/156·91	**0·000/0·000**	1/1	8·02/18·60	**0·007/0·000**
Offspring type	4/1	11·48/39·33	**0·000/0·000**	4/1	3·39/8·80	**0·015/0·004**	4/1	6·12/7·11	**0·000/0·010**
Population type	1/1	0·69/0·91	0·410/0·342	1/1	0·96/0·36	0·331/0·551	1/1	0·02/0·22	0·896/0·643
Parent combination	1/1	4·06/6·05	**0·047/0·016**	1/1	1·97/0·57	0·166/0·425	1/1	0·00/0·82	0·989/0·368
Offspring type × Temp.	4/1	1·12/0·01	0·355/0·938	4/1	2·90/3·73	**0·029/0·057**	4/1	2·83/6·66	**0·034/0·012**
Population type × Temp.	1/1	1·89/1·12	9·173/0·268	1/1	1·03/2·52	0·314/0·116	1/1	1·06/0·02	0·308/0·895
Parent comb. × Temp.	1/1	1·44/0·80	0·234/0·374	1/1	0·28/0·07	0·599/0·799	1/1	0·40/0·18	0·531/0·676
Offspr. type × Popul. type	4/1	1·83/2·97	0·131/0·088	4/1	1·82/0·96	0·137/0·329	4/1	2·00/0·00	0·110/0·981
Parent comb. × Popul. type	1/1	0·01/0·02	0·915/0·894	1/1	0·61/0·09	0·437/0·767	1/1	0·01/0·30	0·924/0·588
Remaining tadpoles	1/1	26·48/23·79	**0·000/0·000**						
Error	81/99			60/77			49/64		

Figures

Figure 1: Locations of sampled populations near Kyritz (Germany), Hellberg (Switzerland) and Šaštín-Stráže (Slovakia) with the following air-line distances between them: Kyritz – Hellberg 680 km, Kyritz – Šaštín-Stráže 585 km, Hellberg – Šaštín-Stráže 640 km.

Figure 2: Proportions of surviving tadpoles in relation to parent combination (a) and temperature (b), and days to metamorphosis (c) and weight at metamorphosis (d) in relation to temperature. Shown are means with standard errors. H-H: both parents are hybrids, H-P: one parent is a hybrid (LLR, LR or LRR) and the other from a parental species (LL or RR).

Figure 3: Proportions of surviving tadpoles (a), days to metamorphosis (b) and weight at metamorphosis (c) in relation to offspring genotype. Shown are means with standard errors. On the left side of each figure, the five categories of genotypes are plotted separately; on the right side they are grouped into two categories: hybrids (LLR, LR and LRR pooled) and parents (LL and RR pooled, including two LLL and one RRR in Figure 3a). In figures b) and c) results are also separated by temperature, because of the significant genotype x temperature interaction (see Table 4).

Figure 1

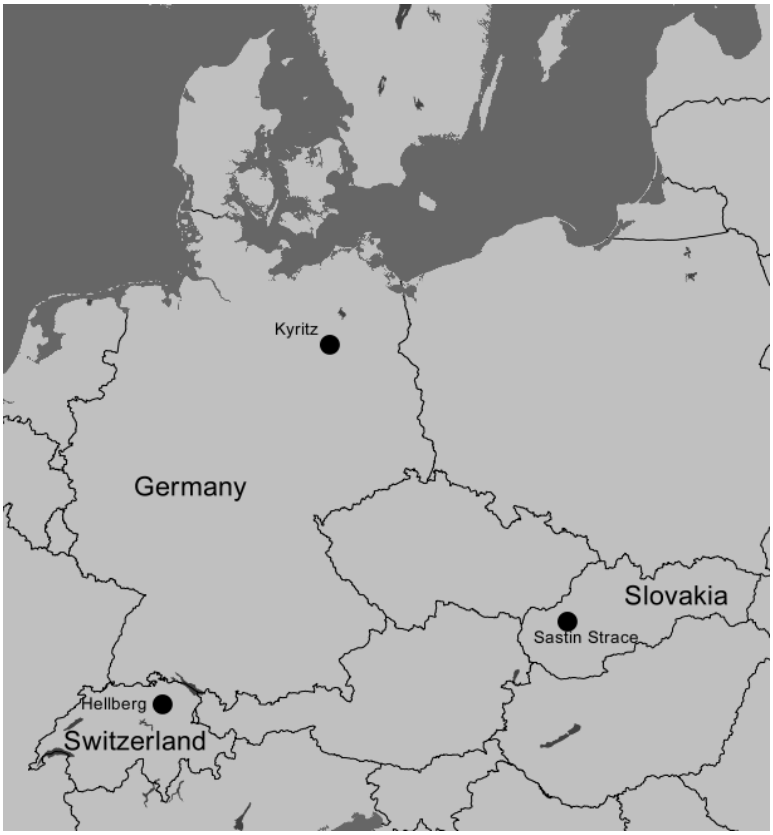

Kyritz
Germany
Slovakia
Sastin Strace
Hellberg
Switzerland

Figure 2

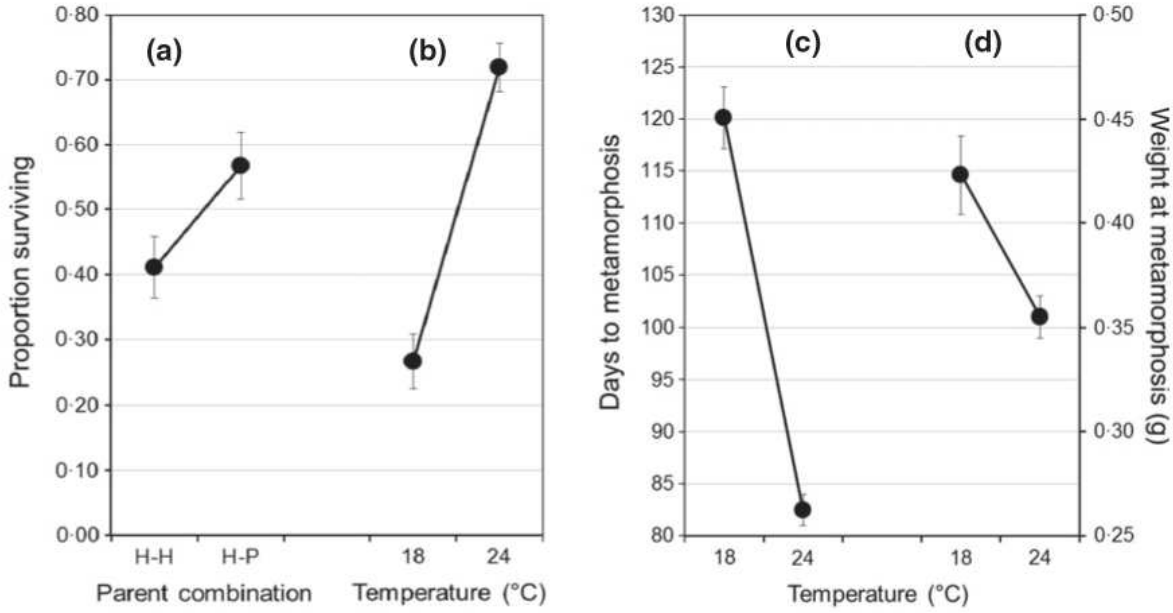
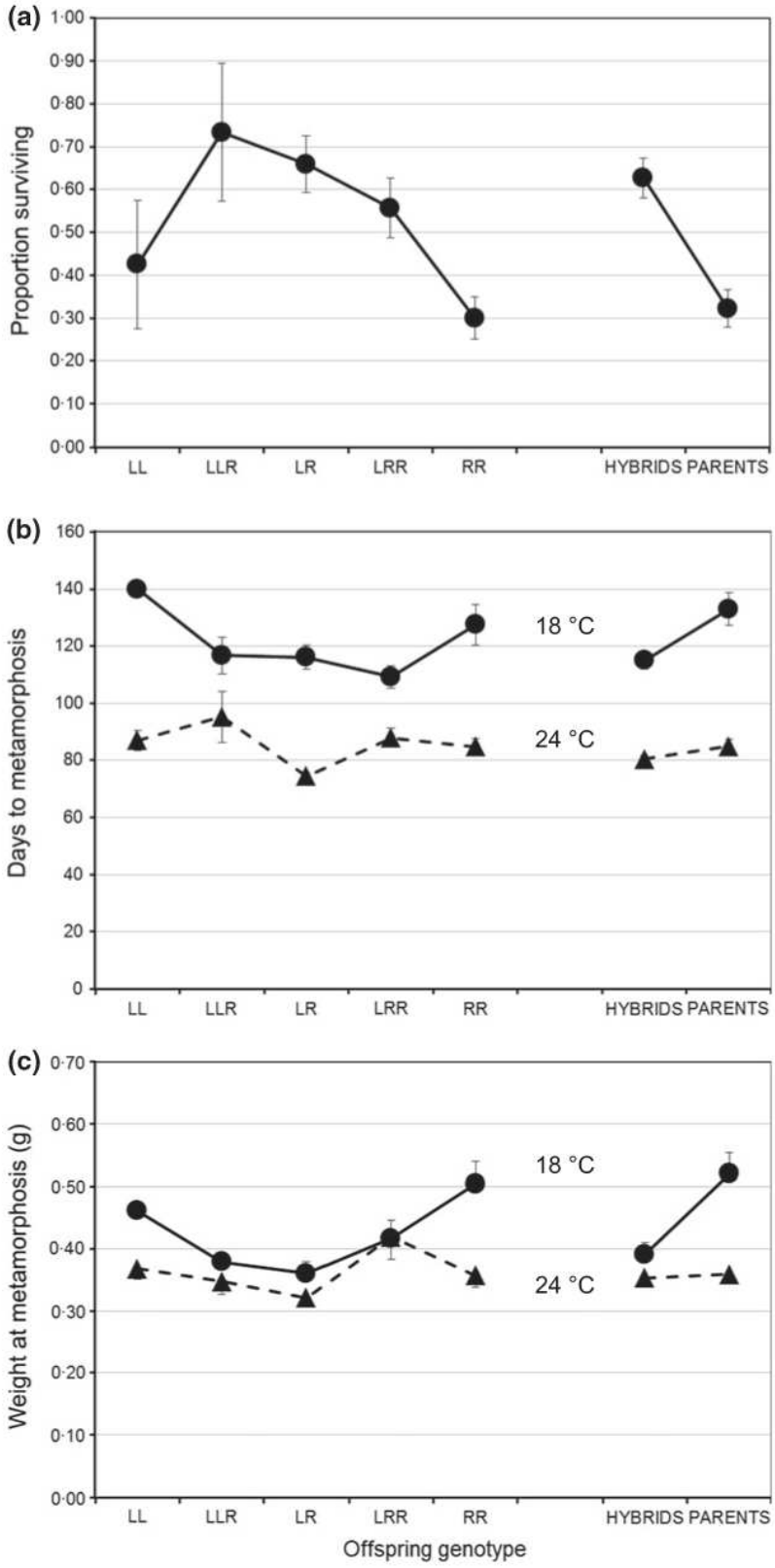

(a)
(b)
(c)
(d)
Proportion surviving
0·80
0·70
0·60
0·50
0·40
0·30
0·20
0·10
0·00
H-H
H-P
Parent combination
18
24
Temperature (°C)
Days to metamorphosis
130
125
120
115
110
105
100
95
90
85
80
Weight at metamorphosis (g)
0·50
0·45
0·40
0·35
0·30
0·25
18
24
Temperature (°C)

Figure 3


(a)
Proportion surviving
1·00
0·90
0·80
0·70
0·60
0·50
0·40
0·30
0·20
0·10
0·00
LL
LLR
LR
LRR
RR
HYBRIDS PARENTS
(b)
Days to metamorphosis
160
140
120
100
80
60
40
20
0
18 °C
24 °C
LL
LLR
LR
LRR
RR
HYBRIDS PARENTS
(c)
Weight at metamorphosis (g)
0·70
0·60
0·50
0·40
0·30
0·20
0·10
0·00
18 °C
24 °C
LL
LLR
LR
LRR
RR
HYBRIDS PARENTS
Offspring genotype

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Genetic and cytogenetic characteristics of pentaploidy in water frogs

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Abstract.- We describe a pentaploid froglet (LLLRR; 3 *Pelophylax lessonae* and 2 *Pelophylax ridibundus* genomes) that has never been reported before within the water frog (*Pelophylax esculentus*) hybrid complexes. The pentaploid specimen was found among almost all-triploid siblings obtained from a diploid female *P. esculentus* (LR) crossed with a diploid male *P. lessonae* (LL). We confirmed ploidy levels of the parents and the offspring by karyotyping, microsatellite analysis (18 loci) and measurements of DNA content and erythrocyte size. Microsatellite analysis indicated that the pentaploid originated from a tetraploid ovum (LLRR) fertilized by a haploid sperm (L). Surprisingly, the erythrocytes of the pentaploid were not proportionally larger than in triploids, despite a higher DNA content. Only 6.7% of its erythrocytes were distinctly large, whereas the others varied strongly in shape and size; besides typical ovoid mature erythrocytes there were small, tear-shaped or enucleated ones. We discuss the possibility of loss of some cytoplasm by large erythrocytes as a result of mechanical damages during circulation through the narrow vessels; when the erythrocytes achieve a relatively higher surface-to-volume ratio, they may function more effectively in a proper gas exchange.

Keywords: Cells size; enucleated erythrocytes; genome size; hybridogenesis; microsatellites; *Pelophylax esculentus*, polyploidy.

Introduction

The water frog, *Pelophylax esculentus* (genus *Rana* until Frost et al. 2006), is a natural bisexual hybridogenetic hybrid between the pool frog, *P. lessonae* (genotype LL), and the marsh frog, *P. ridibundus* (RR; for review, see Graf and Polls-Pelaz 1989). Both species and the hybrid can be freely crossed artificially, giving progeny of various viability (Berger 1988, Berger et al. 1994). In most of its European range, *P. esculentus* appears as a diploid form (LR) and lives in sympatry with one of the parental species. In some populations, diploid hybrids coexist with allotriploid hybrids (LLR and/or LRR), especially in central and northern parts of the species complex range (Mikulíček and Kotlík 2001, Rybacki and Berger 2001, Borkin et al. 2004, Plötner 2005). Moreover, in the northern part of the range (Sweden, Denmark, Northern Germany, Northern Poland), diploids and triploids often form all-hybrid populations (Christiansen and Reyer 2009, Arioli et al. 2010). Spontaneous cases of tetraploidy were reported in some populations, but it is not known if they are of any importance for the genetic dynamic of these populations (Borkin et al. 2004, Christiansen 2009). This study is the first report describing a single case of pentaploidy in hybridogenetic water frogs that arose among almost all-triploid offspring obtained from a *P. esculentus* female crossed with a *P. lessonae* male.

It is well documented that DNA content is strongly and positively correlated with erythrocyte size in different groups of vertebrates (Gregory 2001, Sessions 2008). The clearest demonstration of positive correlation between genome size and cell size are polyploids that result from the duplication of entire chromosome sets (Gregory 2003, Mable et al. 2011). Cell size, however, is not always directly proportional to genome size, as is observed in animals with higher ploidy levels (Mable et al. 2011). Erythrocytes in polyploid amphibians are proportionally bigger than in diploids but are often less than a theoretical factor (for example less than two in tetraploids), as might be expected with the duplication of entire chromosome sets (Deparis et al. 1975, Matson 1990, Mable et al. 2011). Furthermore, in some research authors have reported that the proportion of abnormal erythrocyte shapes (dumbbell-shaped, u-shaped, tear shaped and round) increased with

increasing ploidy levels (Liu et al. 2003, Lu et al. 2009). An unusually high percentage of atypical enucleated erythrocytes (> 80%) – probably caused by an extremely large genome size – were described in plethodontid salamanders of the genus *Batrachoseps* (Villolobos et al. 1988, Mueller et al. 2008). The examples mentioned above may demonstrate a relationship between large genomes and the occurrence of abnormal erythrocytes that have originated independently in two different groups of animals (in polyploids and in plethodontid salamanders). Abnormal erythrocytes may result from a random breakage of large cells during circulation, causing the loss of some cytoplasm or even nuclei (Villolobos et al. 1988). Moreover, smaller, more variable in shape, or enucleated erythrocytes, besides having reduced problems with circulation, have a more favorable surface-to-volume ratio that could be advantageous in a proper gas exchange.

In this study we compared erythrocyte sizes of the pentaploid and its triploid siblings raised under the same laboratory conditions. Such an approach reduced the possible influence of environmental factors, which can mask a potential ploidy effect. Measurement of erythrocytes size is also the easiest and reliable method for distinguishing diploids from triploids in *P. esculentus* complex (Günther 1977, Berger et al. 1978, Polls Pelaz and Graf 1988). The results obtained by Kierzkowski (2004) in a large sample of water frogs with known genotypes (110 diploids and 64 triploids) demonstrated that discrimination between diploid and triploids was possible for 99.4% individuals. Furthermore, the pentaploid froglet (as revealed by karyotyping) provided an opportunity to test the efficiency of microsatellite analysis in identifying higher ploidy levels of water frogs. Molecular analysis also allows to determine unambiguously the participation of each of the parents in term of ploidy and genomic composition of their gametes.

The objective of our research was to characterize the pentaploid froglet in comparison to its triploid siblings, which enabled us (1) to examine the effect of a major increase in DNA content on erythrocyte size, and (2) to examine the efficiency of microsatellite analysis and measurements of erythrocyte size in correct identification of pentaploidy in water frogs.

Material and methods

Animals and crossing experiments

In a larger project, aimed at comparing growth and metabolic rates in diploid (2n) and triploid (3n) frogs, we crossed altogether 16 pairs of male *P. lessonae* and diploid female *P. esculentus* to obtain a high frequency of 3n offspring (Czarniewska et al. 2011). In one of the crosses one pentaploid (5n) froglet occurred. The father derived from a mixed *lessonae-esculentus* population (Rogaczewo Wielkie, Poznań district, Poland, 52º02'23"N, 16º50'21"E; datum = WGS84) and the diploid mother derived from an all-hybrid *esculentus-esculentus* population (Wysoka Kamieńska, Szczecin district, Poland, 53º49'18"N, 14º50'37"E). We determined ploidy and genome compositions of the parents on the basis of morphological indices and erythrocyte size, and confirmed them later by microsatellite analysis. To obtain offspring, we used artificial fertilization, stimulating the female with the luteinizing salmon hormone (LHRH, Bachem Bioscience Inc.; Berger et al. 1994). We obtained sperm suspension directly from testes, dissected from the anaesthetized male (MS 222).

To obtain the highest percentage of triploid offspring, we took only eggs classified as large (98.7% of the whole clutch) for further rearing. When tadpoles began to feed (stage 25; Gosner 1960) we selected 40 healthy-looking individuals for further rearing. Among the 21 individuals, which completed metamorphosis, only seven (lab numbers 13/09, 15/09, 34/09, 35/09, 45/09, 51/09, 52/09) survived the next three months, when we sacrificed them for analyses. We kept tadpoles and juvenile frogs under laboratory conditions with controlled temperatures (24ºC and 23ºC, respectively) and natural photoperiods. All animals were fed *ad libitum*.

Karyotyping

We carried out karyotyping for all seven survivors. A day before tissue preparation we injected each animal peritoneally with 0.5 mL of 0.3% colchicine (Sigma). To obtain metaphase plates, we squashed inner intestine epithelial tissue fragments under a cover slip in a drop of 70% acetic acid. We stained the chromosomes using the AMD/DAPI method (fluorescence double-

staining technique), which enables discrimination between R and L chromosomes due to the fluorescence of AT-rich pericentromeric heterochromatin regions in R chromosomes (Heppich et al. 1982). We used chromosomes of the 10^{th} pair, which were easily distinguishable by their secondary constriction (NOR region), as a genome composition marker (Ogielska et al. 2004). Chromosome counting was done on 3-5 complete metaphase plates from each individual. We examined the slides using a Nikon (Eclipse E600) microscope equipped with a fluorescence lamp with appropriate filters. The microscope was connected to the digital camera.

Measurements of erythrocyte size and DNA content

We made blood smears from a cut finger tip on microscopic slides, air-dried them for one hour and stored in darkness at room temperature. We acquired images of the erythrocytes using microscope (Zeiss Jena) with a camera connected to a computer equipped with the Multiscan CSS computer program. Long and short axes of 30 randomly chosen undamaged ellipsoid erythrocytes were measured for each individual. We calculated the erythrocytes' area from the $ab\pi / 4$ formula, where a and b denote long and short axes, respectively.

We measured DNA content in erythrocyte nuclei of the pentaploid, in one triploid sibling, and three adult *P. lessonae* (diploid genome) as a reference. We stained blood smears of all specimens in the same series according to Feulgen's method with Schiff reagent (Ogielska et al. 2004). Images of the manually selected erythrocyte nuclei were acquired with a standard video camera, optically coupled with an upright Zeiss Axioskop 20 microscope and processed by the computer image analysis system KS400 (Carl Zeiss Vision).

We calculated the Integrated Optical Density (IOD) for each analyzed nucleus according to the formula: $IOD = S \times D$, where S is the area of the nucleus, and D is optical density.

Optical density is expressed as: $D = \log I_0 / I_1$, where I_0 represents mean background brightness, and I_1 is mean brightness of the nuclei.

For each smear, we measured 60 undamaged and properly stained nuclei and calculated mean IOD values.

Analysis of the size of erythrocytes

We tested normality of distributions of erythrocyte area in each specimen with the Shapiro-Wilk test. Since some measurements showed nonnormal distributions (in the pentaploid and one triploid), we did not compare centers of distribution and tested the differences in dispersion of erythrocyte sizes between pentaploid and every single triploid using Aly's permutation test (for details see Good 2005). To combine significance levels (p-values) obtained from six pairs of comparisons, we used Jost's formula (Jost 2008). It gave us the true probability that a set of p-values was produced by chance. We calculated these statistics using Rundom Pro 3.14 software (Jadwiszczak 2009).

Microsatellites

We analyzed nine individuals altogether (two parents and seven of their offspring). The starting material consisted of toes fixed in Ethanol 80º (parents) and dry muscle with no fixation (offspring). We extracted DNA using the Qiagen BiosprintTM 96 DNA Blood Kit following the supplier's protocol.

We used a set of 18 microsatellite primer pairs run in four primer mixes (Primer Mix 1A (PM1A): CA1b6, Ga1a19 redesigned (Arioli et al. 2010), RlCA1b5, RlCA5 (Garner et al. 2000), Rrid064A (Christiansen and Reyer 2009) ; Primer Mix 1B (PM1B): Re2CAGA3 (Arioli et al. 2010), Res16, Res20 (Zeisset et al. 2000), RlCA2a34 (Christiansen and Reyer 2009) ; Primer Mix 2A (PM2A): ReGA1a23, Rrid169A, Rrid059A redesigned (Christiansen and Reyer 2009), Res22 (Zeisset et al. 2000), Rrid013A (Hotz et al. 2001); Primer Mix 2B (PM2B): Re1Caga10 (Arioli et al. 2010), RlCA18 (Garner et al. 2000), RlCA1a27, Rrid135A (Christiansen and Reyer 2009)). We run PCR products for fragment length analysis on an ABI 3730 Avant capillary sequencer with internal size standard (GeneScan-500 LIZ) and scored the alleles with the GeneMapper software v3.7 (Applied Biosystems).

Loci Res20, RlCA1a27 and RlCA18 were species-specific for *P. lessonae*, and Re2CAGA3, Res22 and Rrid169A were species-specific for *P. ridibundus*. The other 12 loci amplified in both *lessonae* and *ridibundus* genomes (Christiansen 2005, Christiansen 2009, Arioli et al. 2010).

Seven of the primers showed a genome dosage effect that enables the detection of the presence of multiple copies of an allele in the genome by comparing the ratio of the heights of the peaks (allele 1: allele 2; Christiansen 2005). We then corrected the ratio by the slope between the top of each peak obtained for a diploid specimen. In order to assess the ploidy level of the specimens and to determine their genomic composition, we plotted the values against expected values of allele ratios of 1 to 1 (expected ratio = 1 for diploids), 1 to 2 and 2 to 1 (0.5 and 2 for triploids), or 3 to 2 (1.5 for pentaploids). When the number of values allowed to get an estimate of the variance we have run a one tailed one sample t-test, using the program R (version 2.15.1, R Development Core Team 2012), to test if there was a significant difference between the value of the corrected ratio of the putative pentaploid and the mean of the diploid or triploid values. Following the microsatellite alleles from the parents to the offspring allowed us to unequivocally determine the contribution of each parents in term of genome composition and of ploidy of the gametes they produced.

Results

Karyotyping

Among seven analyzed offspring one was pentaploid with 65 chromosomes, other froglets were triploids with 39 chromosomes. We determined the genome composition on the basis of fluorescent pericentromeric regions of the 10^{th} pair of chromosomes. We classified all triploids as LLR and the pentaploid as LLLRR (Figure 1).

Erythrocyte size and DNA content

Contrary to expectations, erythrocytes of the pentaploid individual were not proportionally larger than those in triploids; median values of area in the 5n individual were actually lower (Figure 2). The distributions of erythrocyte area in the pentaploid deviated significantly from normality ($W = 0.92$, $p = 0.031$); distribution was positively skewed with long right “tails” representing the biggest cells.

We found a significantly higher dispersion of erythrocytes area in the 5n animal in comparison with every single triploid (at significance level $\alpha = 0.05$; Figure 2). The probability that such a set of p-values was produced by chance was lower than 0.0001. Erythrocytes area in the pentaploid ranged between 115–558 μm^2, and between 252–392 μm^2 in triploids. The size of 30% of erythrocytes in the pentaploid were not uniform and formed two extremes: large erythrocytes (area > 400 μm^2), and small erythrocytes (area range: 115–165 μm^2) that represented 6.7% and 23.3% of all erythrocytes, respectively. The area of the remaining 70% of ellipsoid erythrocytes ranged between 188–392 μm^2.

The most striking feature of the pentaploid blood was the variety of unusual shapes of erythrocytes (Figure 3). We observed tear-shaped, dumbbell-shaped and U-shaped cells and enucleated cells.

Despite the variation of shapes, DNA content in all blood cells was at the pentaploid level. The IOD value for the diploid genome (2C), measured for three *P. lessonae*, averaged 4.40. Therefore a theoretical IOD value for the haploid genome (1C) would be 2.20. As expected, the measured IOD value for the triploid specimen (No 52/09) was 6.86 (3C), and for the pentaploid specimen 10.94 (5C).

Microsatellites

After successful amplification, the 18 microsatellite loci gave complete multilocus genotypes for the two parents and seven offspring (Table 1).

Microsatellite analysis was consistent with the fact that among the seven offspring analyzed, six were triploid (LLR) while one specimen was a LLLRR pentaploid. We showed that the *P. lessonae* father was a typical haploid gamete donor, whereas the *P. esculentus* mother passed, in the case of the specimen 51/09, two copies of each of her L and R genomes (LLRR). We detected triploidy in the offspring directly, by the number of peaks for at least three loci displaying three peaks in the PCR product analysis. Pentaploidy could not be directly detected by the number of allele peaks because both parents were diploid and the alleles transmitted in more than one copy would give only one and the same peak in the fragment length analysis (Figure 4). Hence, the microsatellite peaks only showed that the

P. lessonae father donated a haploid L sperm, while the *P. esculentus* mother produced some eggs carrying both the L and R genomes, but with an unknown number of copies. Only the ratio of the heights of peaks for the alleles with dosage effect can provide information on the number of copies. Seven of the analyzed loci (CA1b6, Ga1a19, Res16, Res20, RlCA2a34, ReGA1a23 and Rrid059) displayed the genome dosage effect. In each locus, such comparison of the ratio can only be made between the same pair of alleles (e.g. in Table 1, for alleles 120 and 124 in loci Res20, the comparison could only be made between specimen 51/09 and two other siblings).

We were able to compare the mother and its seven offspring in the case of loci Res16, Ga1a19 and CA1b6, using the peak height ratios of the alleles 121 (L specific) and 127 (R specific), 195 (L) and 201 (R), and 78 (L) and 92 (R), respectively. Here we only present details for the one locus, but the results of the other loci are fully congruent with the following conclusion. The corrected height ratio for alleles 121 and 127 in locus Res16, for six offspring specimens (13/09, 15/09, 34/09, 35/09, 45/09, 52/09), gave values clustering around 2, with a 99% confidence interval of 1.901 ± 0.086, while the offspring specimen 51/09 showed a significantly different value of 1.503 (one sample t-test $t(5) = 18.541$, $p < 0.001$; Figure 5). This result allows us to conclude that six individuals carried two copies of allele 121 for one copy of allele 127, while specimen 51/09 had three copies of allele 121 for two of alleles 127. This example, together with the congruent results of other loci, allowed us to unequivocally determine that the male always provided haploid L sperm that – combined with diploid LR ova results in the triploid offspring, and with a tetraploid LLRR ovum results in the pentaploid specimen.

Discussion

Pelophylax esculentus is a bisexual hybrid that reproduces by hybridogenesis; in this process one of the parental genomes is eliminated before meiosis, and gametes produced by a hybrid are clonal (L or R); when fertilized by gametes of a related species, the progeny is semiclonal (Graf and Polls-Pelaz 1989). One of the well-documented phenomena observed in *P. esculentus* is the production of eggs of various sizes (small, medium, and large), ploidy, and genome composition by hybrid females (Berger 1988, Czarniewska et al.

2011). As a rule, large eggs are diploid and give rise to triploid progeny, as was also confirmed in our study. Until now it was thought that diploid *P. esculentus* females from all-hybrid populations produce mostly R and LR eggs (Christiansen 2009). We showed that one female also produced a tetraploid LLRR egg that gave rise to the pentaploid offspring. Such genome composition of a gamete may originate from suppression of both meiotic divisions and retention of all tetrads in a single nucleus, or suppression of the second division in an already tetraploid oocyte I, which might be a consequence of chromosomal reduplication (Fankhauser 1945, Sessions 1982, Otto and Whitton 2000). The pentaploid individual developed properly as a tadpole and then as a froglet and displayed no abnormalities characteristic of water frog progeny (Berger 1967, Ogielska 2009). Unfortunately, we detected the unusual ploidy level only after the death of the animal and for this reason we did not raise the pentaploid further to see how long it could survive and which type of gamete it could produce.

Triploid water frogs have significantly larger (30–50%) erythrocytes than diploids, a characteristic which is often used for preliminary assessment of ploidy (Günther 1977, Polls Pelaz and Graf 1988). We expected that the pentaploid specimen would have significantly larger erythrocytes than their triploid siblings, but instead we found a high variability of their shapes and sizes and thereby, the mean value of area of the cells was lower than in triploids. Despite their different sizes and shapes, DNA content measurements indicated 5C values in all analyzed erythrocytes in the pentaploid frog, and we thereby excluded tissue mosaicism, as was the case of 1n/3n erythrocytes described in *P. esculentus* by Berger and Ogielska (1994). We also excluded the possibility that abnormal erythrocyte shapes observed in the pentaploid froglet were artifacts formed during the blood smear because within hundreds of smears of diploids and triploids only the pentaploid specimen had such abnormalities. More variable or generally smaller erythrocyte sizes than expected from theoretical calculations were also observed in pentaploid newts of the genus *Pleurodeles*, tetraploid *Hyla versicolor* and tetraploid *Bufo viridis* (Deparis et al. 1975, Matson 1990, Stöck and Grosse 1997). Nevertheless, the mean size of pentaploid *Pleurodeles* newt cells was significantly bigger than triploid ones, and slightly bigger than tetraploid ones. Another

observation was that 5n newts do not regulate hematocrit and hemoglobin values and are slightly anemic (Deparis et al. 1975). We conclude that the mean size of erythrocytes is not a good marker for determination of pentaploidy in water frogs, but high variability of the cells sizes and shapes could be a strong clue for the presence of ploidy level higher than 3n.

Enucleated erythrocytes, extremely unusual among nonmammalian vertebrates, have been reported in plethodontid salamanders of the genus *Batrachoseps*. All species of this genus have miniaturized or attenuated body form and large genome size in comparison with other vertebrates (Villolobos et al. 1988). Mueller et al. (2008) suggested that such cells may have evolved in response to a physical constraint against the circulation of large, fully nucleated cells through the circulatory system of a miniaturized/attenuated animal. The presence of similar unusual tear-shaped, very small and enucleated erythrocytes in the peripheral blood of the pentaploid *P. esculentus* described herein suggests that mechanism of their formation is not necessarily adaptive and might be purely mechanical, as was proposed by Villolobos et al. (1988). We believe that during circulation through narrow vessels, large erythrocytes may become deformed, and after that may lose some cytoplasm in the process of amitotic division, as was proposed by Emmel (1924) and supported by Cohen (1982). Gibiński's (1947) observations of the origin of enucleated and tear-shaped cells from mature erythrocytes in water frogs under anoxemia conditions might confirm the above assumption.

Concerning the suitability of the different loci to detect pentaploidy, we cannot recommend any specific loci. Their ability to detect higher ploidy levels directly depends on the genotype of the parents, when analyzing some offspring, and/or on the heterozygosity of the specimens, when analyzing population genetics data (Ramsden et al. 2006). Nevertheless we advise researchers who use microsatellites to define the genome composition of water frogs to carefully check their data from a genome dosage effect point of view. By calculating and plotting the different peak height ratios for all combinations of alleles (if possible normalized by dividing them by the same ratio for a diploid individual, and keeping in mind that those comparisons can only be made for the same pair of alleles), one could find clustering values corresponding to different genomic compositions. The large spread of such

ratios with discrete groups is a very strong clue for the presence of different ploidy types. Authors of previous studies detected and separated LR diploids (with an L:R ratio of 1) from two types of triploids (LLR and LRR) easily, by showing that diploids cluster in a discrete group in-between the two other clusters formed by LLR and LRR frogs (with ratios of 2 and 0.5, respectively; Christiansen 2005, 2009, Christiansen and Reyer 2009, 2011, Arioli et al. 2010). Here we show that pentaploid individuals can be detected in the same way, but that that their ratio of 1.5 (respectively 0.67) places them in-between diploid and triploid categories, with the risk of blurring the clusters when looking at a large number of frogs.

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Table

Table 1: Microsatellite multilocus genotypes of the two parents and of the seven offspring; Pl. indicates the ploidy; Gen. indicates the specific genotype composition; A1, A2 and A3 indicate first, second and third alleles respectively. *P. lessonae* specific alleles are in italics and *P. ridibundus* specific alleles are nonitalicized. Loci Re2Caga3, Rrid169A and Res22 are monomorphic for the R alleles 212, 189 and 116 respectively, in all specimens except for the father. Locus RICA1a27 is monomorphic for the L allele 111 in all specimens.

			CA1b6			Ga1a19		RlCA1b5		RlCA5			Rrid064A		RlCA2a34			Res16		Res20		ReGA1a23			Rrid059A			Rrid013A		Re1Caga10		RlCA18		Rrid135A	
Sample	Pl.	Gen.	A1	A2	A3	A1	A2	A1	A2	A1	A2	A3	A1	A2	A1	A2	A3	A1	A2	A1	A2	A1	A2	A3	A1	A2	A3	A1	A2	A1	A2	A1	A2	A1	A2
Male	2N	LL	78	82		*195*		*118*		256	*260*		*242*		*140*	*156*		*121*		*120*	*126*	*123*	*133*		*278*			296		97		*179*	*186*		
Female	2N	LR	78	92		*195*	201	*118*	132	232	256		225	*242*	106	*145*		*121*	127	*124*		98	*125*		*280*	315		281	*296*	97	135	*191*		203	*236*
13/09	3N	LLR	78	92		*195*	201	*118*	132	232	256		225	*242*	106	*145*	*156*	*121*	127	*124*	*126*	98	*123*	*125*	278	*280*	315	281	*296*	97	135	*179*	*191*	203	*236*
15/09	3N	LLR	78	82	92	*195*	201	*118*	132	232	256	*260*	225	*242*	106	*140*	*145*	*121*	127	*120*	*124*	98	*125*	*133*	278	*280*	315	281	*296*	97	135	*186*	*191*	203	*236*
34/09	3N	LLR	78	82	92	*195*	201	*118*	132	232	256	*260*	225	*242*	106	*145*	*156*	*121*	127	*120*	*124*	98	*125*	*133*	278	*280*	315	281	*296*	97	135	*179*	*191*	203	*236*
35/09	3N	LLR	78	92		*195*	201	*118*	132	232	256		225	*242*	106	*145*	*156*	*121*	127	*124*	*126*	98	*125*	*133*	278	*280*	315	281	*296*	97	135	*186*	*191*	203	*236*
45/09	3N	LLR	78	82	92	*195*	201	*118*	132	232	256	*260*	225	*242*	106	*140*	*145*	*121*	127	*124*	*126*	98	*123*	*125*	278	*280*	315	281	*296*	97	135	*179*	*191*	203	*236*
52/09	3N	LLR	78	82	92	*195*	201	*118*	132	232	256	*260*	225	*242*	106	*140*	*145*	*121*	127	*124*	*126*	98	*123*	*125*	278	*280*	315	281	*296*	97	135	*179*	*191*	203	*236*
51/09	5N	LLLRR	78	92		*195*	201	*118*	132	232	256		225	*242*	106	*140*	*145*	*121*	127	*120*	*124*	98	*125*	*133*	278	*280*	315	281	*296*	97	135	*186*	*191*	203	*236*

Figures

Figure 1: Metaphase plates of the pentaploid (5n = 65 chromosomes, genome LLLRR) *P. esculentus* specimens stained with the AMD/DAPI method. Diagnostic chromosomes N^{o} 10, with characteristic secondary constriction, are indicated by arrows.

Figure 2: Statistics of erythrocyte size for triploids and pentaploid *P. esculentus* froglets. UBV = upper box value, 75th percentile; LBV = lower box value, 25th percentile; H = height of the box; nonoutlier values are those located between UBV + 1.5H and LBV + 1.5H; outlier values are greater than 1.5H.

Figure 3: Erythrocytes of the triploid (A) and pentaploid (B) *P. esculentus* froglets. Note the different types of erythrocytes in the pentaploid animal: small (se), tear-shaped (te), enucleated (ee) and large (le).

Figure 4: Amplification patterns of the alleles 121 (L-genome specific) and 127 (R-genome specific) of the microsatellite locus Res16 for (A) a diploid, (B) a triploid, and (C) a pentaploid specimen.

Figure 5: Peak height ratios of a female and the seven polyploid offspring for the microsatellite locus Res16. Plotted are ratios of the height of peak 121 on the height of peak 127, relative to the same ratio in the diploid female which was set to 1. Diamonds are the calculated values, dotted lines represent the expected values for 2n (= 1), 3n (= 2) and 5n (= 1.5).

Figure 1

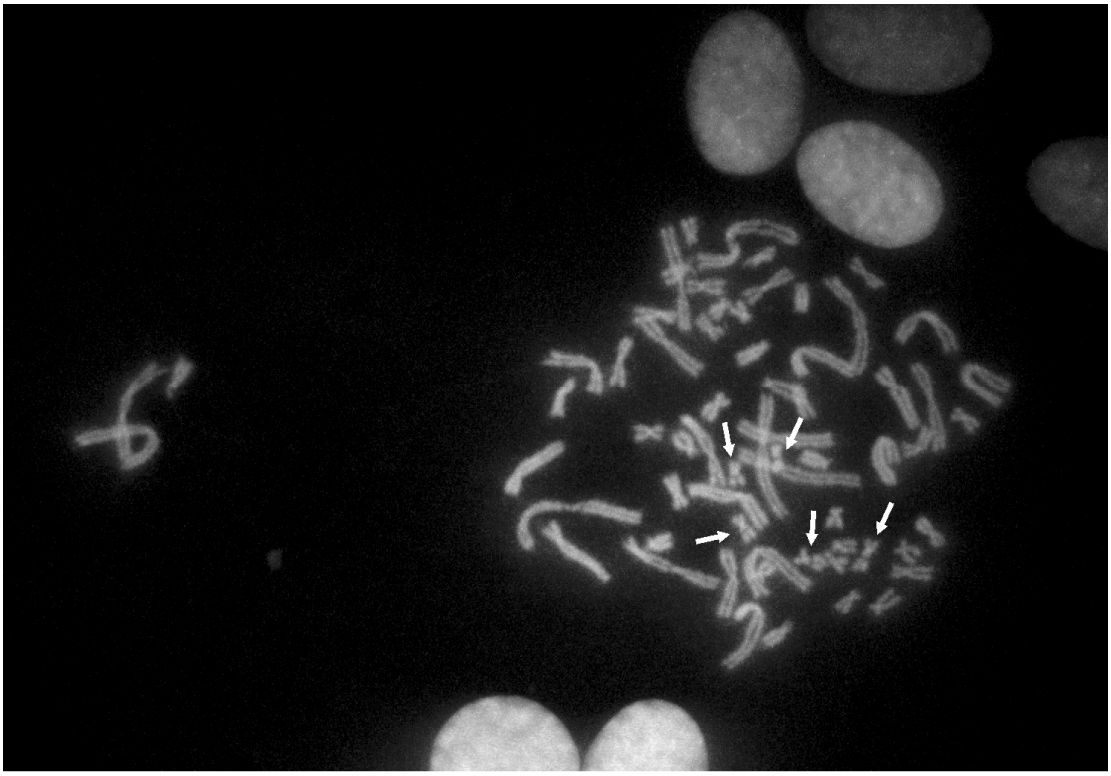


Figure 2

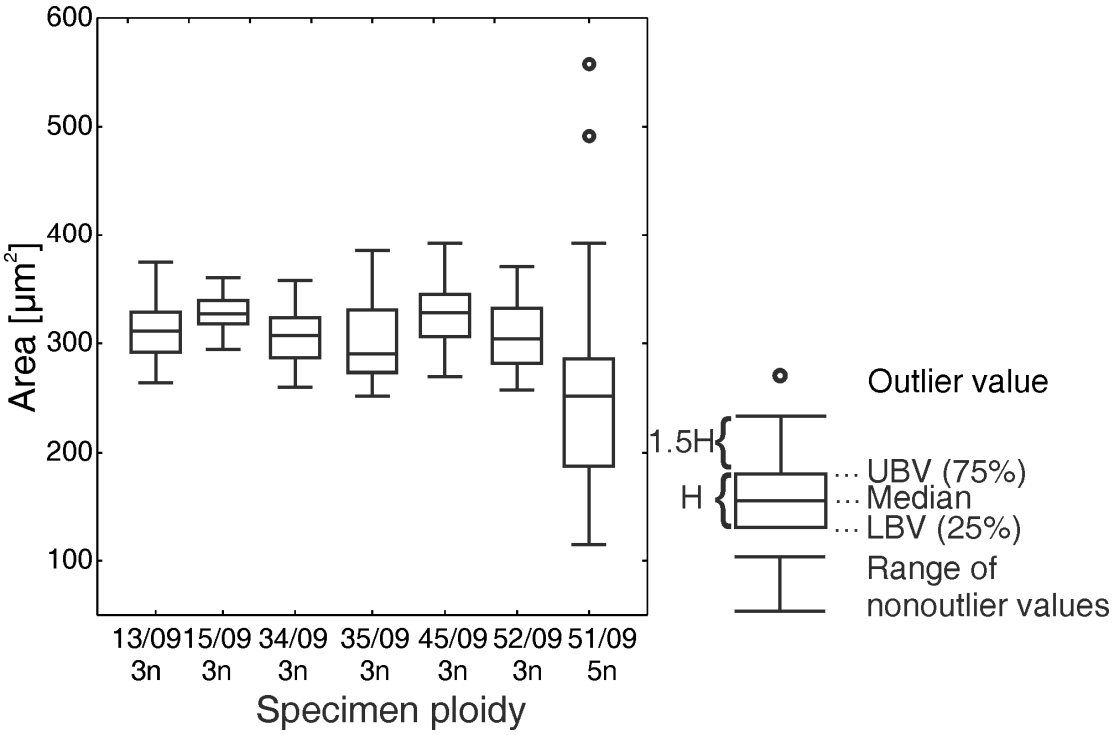

600
500
400
300
200
100
Area [µm²]
13/09 3n
15/09 3n
34/09 3n
35/09 3n
45/09 3n
52/09 3n
51/09 5n
Specimen ploidy
Outlier value
1.5H
H
UBV (75%)
Median
LBV (25%)
Range of nonoutlier values

Figure 3

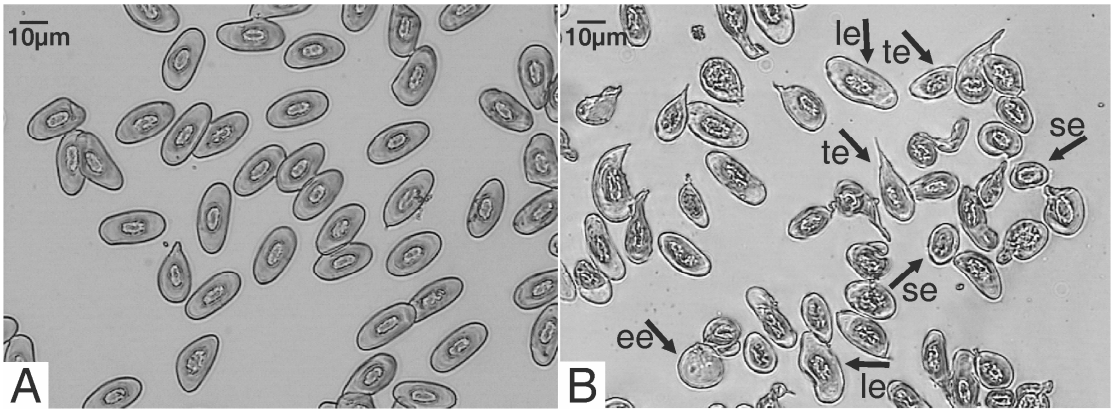


Figure 4

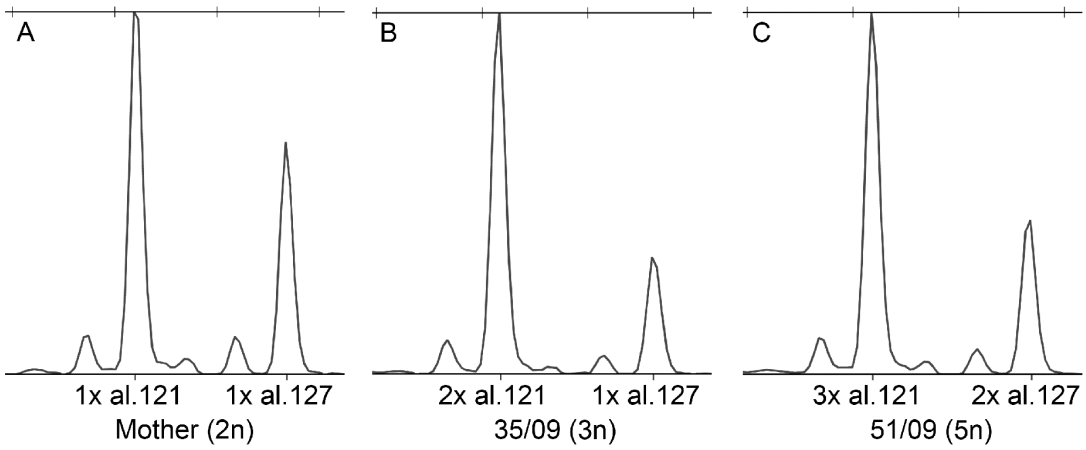
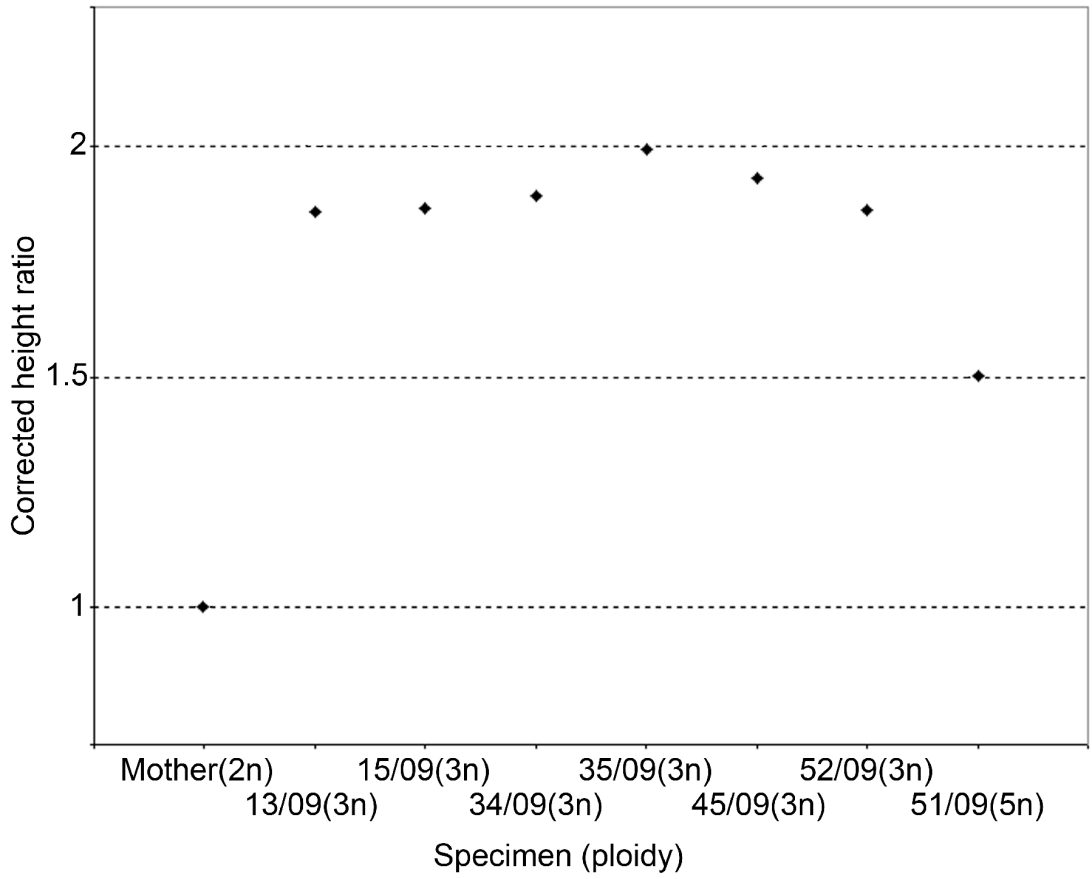

A
1x al.121
1x al.127
Mother (2n)
B
2x al.121
1x al.127
35/09 (3n)
C
3x al.121
2x al.127
51/09 (5n)

Figure 5


Corrected height ratio
2
1.5
1
Mother(2n)
13/09(3n)
15/09(3n)
34/09(3n)
35/09(3n)
45/09(3n)
52/09(3n)
51/09(5n)
Specimen (ploidy)

Appendix

Detailed description of a pentaploid Pelophylax esculentus *froglet using microsatellites*

Here, I present the detailed results of the microsatellites DNA fragments analyses which had to be omitted from the collaborative publication for reasons of brevity.

For each locus I extracted the relevant information relative to the segregation of the genomes at gamete production for the male and the female. Because some loci carried the same alleles in both parents, some conclusions are still putative, but overall none of them led to results that contradicted the proposed pattern of gamete production. In the Tables below, I list the genotypes of the parents and their offspring plus the allele sizes for the genomes of *P. lessonae* (green) and *P. ridibundus* (pink). From these data, I deduce the most likely male and female gamete production patterns, analyzed locus by locus. In cases where a dosage effect allowed the detection of the pentaploid offspring, ratios of the relevant alleles are plotted

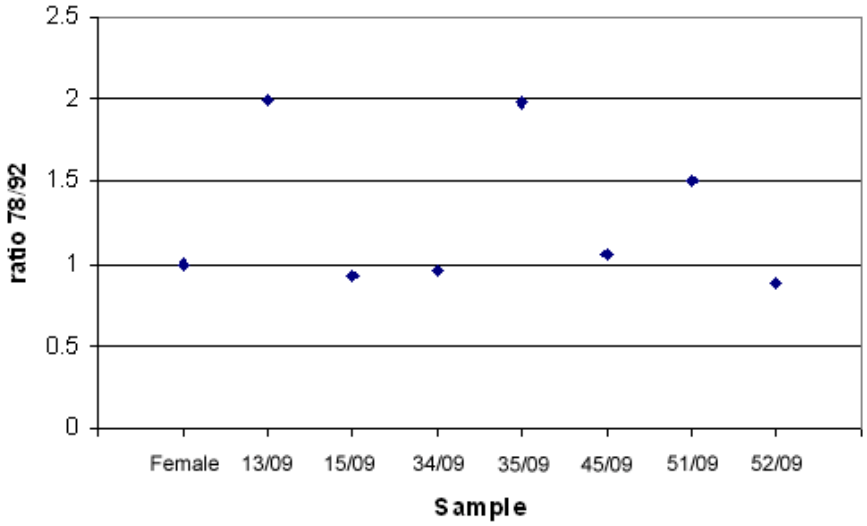
CA1b6:

The *P. lessonae* male possibly produced one L, in this case it shows a mendelian segregation between 78 and 82 (three offspring out of seven inherited allele 78 and four inherited allele 82).

The *P. esculentus* female produced at least LR gametes in four cases and possibly produced the same in the three other cases.

Dosage: Detectable by the ratio height 78 / height 92.

Sample	Sex	Ploidy	Genotype	Expected ratio 78/92	Loci CA1b6 Al. 1	Al. 2	Al. 3
Father	M	2n	LL	-	78	82	
Mother	F	2n	LR	1	78	92	
13/09	M	3n	LLR	2	78	92	
15/09	M	3n	LLR	1	78	82	92
34/09	M	3n	LLR	1	78	82	92
35/09	M	3n	LLR	2	78	92	
45/09	M	3n	LLR	1	78	82	92
51/09	M	5n	LLLRR	1.5	78	92	
52/09	M	3n	LLR	1	78	82	92



RlCA1b5

The *P. lessonae* male possibly produced one L.

The *P. esculentus* female always produced at least one R and possibly an L.

Dosage: No dosage effect detected.

Sample	Sex	Ploidy	Genotype	Loci	
				RlCA1b5	
				Al. 1	Al. 2
Father	M	2n	LL	118	
Mother	F	2n	LR	118	132
13/09	M	3n	LLR	118	132
15/09	M	3n	LLR	118	132
34/09	M	3n	LLR	118	132
35/09	M	3n	LLR	118	132
45/09	M	3n	LLR	118	132
51/09	M	5n	LLLRR	118	132
52/09	M	3n	LLR	118	132

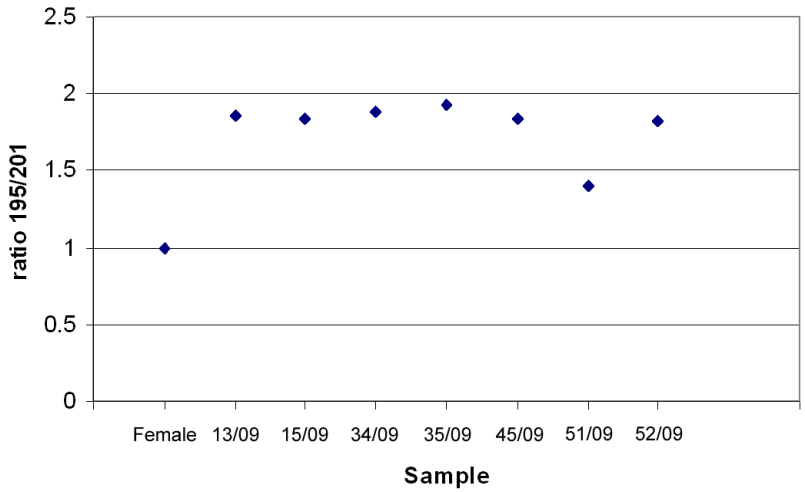
Ga1a19redesigned

The *P. lessonae* male possibly produced one L.

The *P. esculentus* female always produced at least one R and possibly an L.

Dosage: Detectable by the ratio height 195 / height 201.

Sample	Sex	Ploidy	Genotype	Expected ratio 195/201	Loci Ga1a19red. Al. 1	Al. 2
Father	M	2n	LL	-	195	
Mother	F	2n	LR	1	195	201
13/09	M	3n	LLR	2	195	201
15/09	M	3n	LLR	2	195	201
34/09	M	3n	LLR	2	195	201
35/09	M	3n	LLR	2	195	201
45/09	M	3n	LLR	2	195	201
51/09	M	5n	LLLRR	1.5	195	201
52/09	M	3n	LLR	2	195	201



Rrid064A

The *P. lessonae* male possibly produced one L.

The *P. esculentus* female always produced at least one R and possibly an L.

Dosage: No dosage effect detected.

Sample	Sex	Ploidy	Genotype	Loci	
				Rrid064A	
				Al. 1	Al. 2
Father	M	2n	LL		242
Mother	F	2n	LR	225	242
13/09	M	3n	LLR	225	242
15/09	M	3n	LLR	225	242
34/09	M	3n	LLR	225	242
35/09	M	3n	LLR	225	242
45/09	M	3n	LLR	225	242
51/09	M	5n	LLLRR	225	242
52/09	M	3n	LLR	225	242

RlCA5

The *P. lessonae* male possibly produced one L, in this case it shows a mendelian segregation between 256 and 260 (three offspring out of seven inherited allele 256 and four inherited allele 260).

The *P. esculentus* female always produced at least one R and possibly one L.

Dosage: No dosage effect detected.

Sample	Sex	Ploidy	Genotype	Loci		
				RlCA5		
				Al. 1	Al. 2	Al. 3
Father	M	2n	LL	256	260	
Mother	F	2n	LR	232	256	
13/09	M	3n	LLR	232	256	
15/09	M	3n	LLR	232	256	260
34/09	M	3n	LLR	232	256	260
35/09	M	3n	LLR	232	256	
45/09	M	3n	LLR	232	256	260
51/09	M	5n	LLLRR	232	256	
52/09	M	3n	LLR	232	256	260

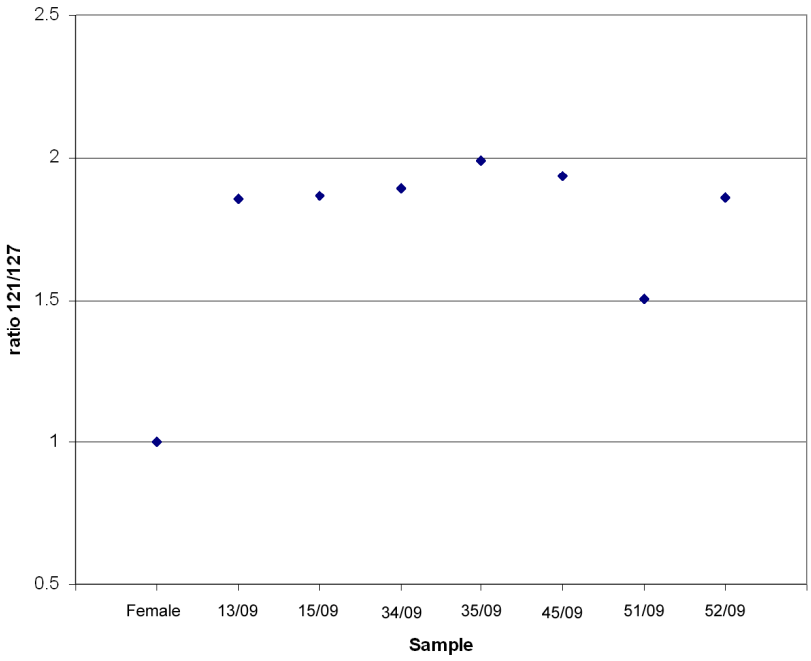
Res16

The *lessonae* male possibly produced one L.

The *esculentus* female always produced at least one R and possibly an L.

Dosage: Detectable by the ratio height 121 / height 127.

Sample	Sex	Ploidy	Genotype	Expected ratio 121/127	Loci Res16 Al. 1	Al. 2
Father	M	2n	LL	-	121	
Mother	F	2n	LR	1	121	127
13/09	M	3n	LLR	2	121	127
15/09	M	3n	LLR	2	121	127
34/09	M	3n	LLR	2	121	127
35/09	M	3n	LLR	2	121	127
45/09	M	3n	LLR	2	121	127
51/09	M	5n	LLLRR	1.5	121	127
52/09	M	3n	LLR	2	121	127



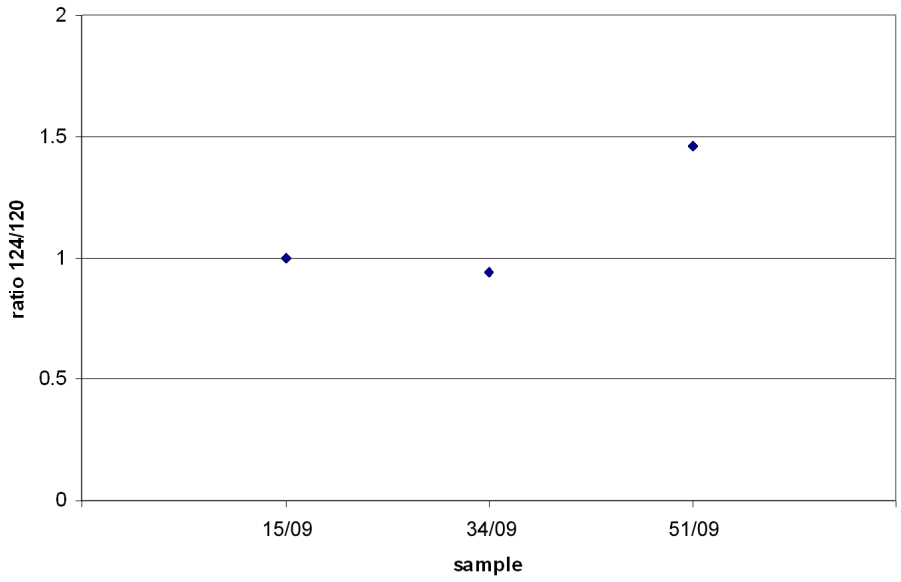
Res20

The *lessonae* male always produced one L, it shows a mendelian segregation between 120 and 126 (three offspring out of seven inherited allele 120 and four inherited allele 126).

The *esculentus* female always produced at least one L and possibly an R (no R specific alleles).

Dosage: Detectable by the ratio height 124 / height 120 (only with three individuals to compare).

Sample	Sex	Ploidy	Genotype	Expected ratio 124/120	Loci Res20 Al. 1	Al. 2
Father	M	2n	LL	-	120	126
Mother	F	2n	LR	-	124	
13/09	M	3n	LLR	-	124	126
15/09	M	3n	LLR	1	120	124
34/09	M	3n	LLR	1	120	124
35/09	M	3n	LLR	-	124	126
45/09	M	3n	LLR	-	124	126
51/09	M	5n	LLLRR	2	120	124
52/09	M	3n	LLR	-	124	126



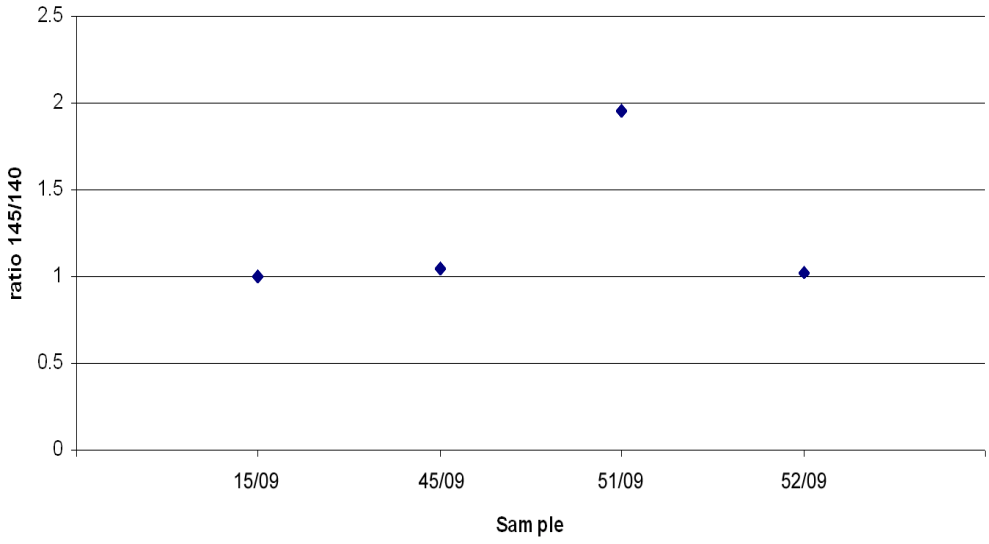
RICA2a34

The *lessonae* male always produced one L, it shows a mendelian segregation between 140 and 156 (four offspring out of seven inherited allele 140 and three inherited allele 156).

The *esculentus* female always produced at least one L and one R (diploid gamete).

Dosage: Detectable by the ratio height 140 / height 145 (only with four individuals to compare).

Sample	Sex	Ploidy	Genotype	Expected ratio 145/140	Loci RICA2a34 Al. 1	Al. 2	Al. 3
Father	M	2n	LL	-	140	156	
Mother	F	2n	LR	-	106	145	
13/09	M	3n	LLR	-	106	145	156
15/09	M	3n	LLR	1	106	140	145
34/09	M	3n	LLR	-	106	145	156
35/09	M	3n	LLR	-	106	145	156
45/09	M	3n	LLR	1	106	140	145
51/09	M	5n	LLLRR	2	106	140	145
52/09	M	3n	LLR	1	106	140	145



Re2Caga3

No information on the *lessonae* male gamete production because of the absence of L specific alleles.

The *esculentus* female always produced at least one R and possibly an L (no L specific alleles).

Dosage: No dosage effect.

Sample	Sex	Ploidy	Genotype	Loci
				Re2Caga3 Al. 1
Father	M	2n	LL	
Mother	F	2n	LR	212
13/09	M	3n	LLR	212
15/09	M	3n	LLR	212
34/09	M	3n	LLR	212
35/09	M	3n	LLR	212
45/09	M	3n	LLR	212
51/09	M	5n	LLLRR	212
52/09	M	3n	LLR	212

Res22

No information on the *lessonae* male gamete production because of the absence of L specific alleles.

The *esculentus* female always produced at least one R and possibly an L (no L specific alleles).

Dosage: No dosage effect.

Sample	Sex	Ploidy	Genotype	Loci
				Res22 Al. 1
Father	M	2n	LL	
Mother	F	2n	LR	116
13/09	M	3n	LLR	116
15/09	M	3n	LLR	116
34/09	M	3n	LLR	116
35/09	M	3n	LLR	116
45/09	M	3n	LLR	116
51/09	M	5n	LLLRR	116
52/09	M	3n	LLR	116

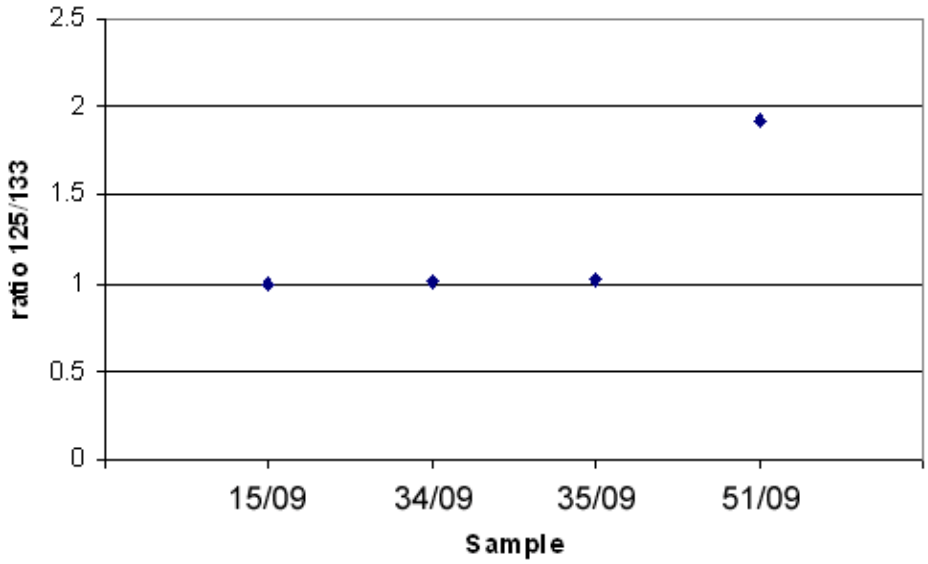
ReGa1a23

The *lessonae* male always produced one L, it shows a mendelian segregation between 123 and 133 (three offspring out of seven inherited allele 123 and four inherited allele 133).

The *esculentus* female always produced at least one L and one R (diploid gamete).

Dosage: Detectable by the ratio height 125 / height 133 (but not in 98/125 or 98/133) (only with four individuals to compare).

Sample	Sex	Ploidy	Genotype	Expected ratio 125/133	Loci ReGa1a23 Al. 1	Al. 2	Al. 3
Father	M	2n	LL	-	123	133	
Mother	F	2n	LR	-	98	125	
13/09	M	3n	LLR	-	98	123	125
15/09	M	3n	LLR	1	98	125	133
34/09	M	3n	LLR	1	98	125	133
35/09	M	3n	LLR	1	98	125	133
45/09	M	3n	LLR	-	98	123	125
51/09	M	5n	LLLRR	2	98	125	133
52/09	M	3n	LLR	-	98	123	125



Rrid169A

No information on the *lessonae* male gamete production because of the absence of L specific alleles.

The *esculentus* female always produced at least one R and possibly an L (no L specific alleles).

Dosage: No dosage effect.

Sample	Sex	Ploidy	Genotype	Loci
				Rrid169A Al. 1
Father	M	2n	LL	
Mother	F	2n	LR	189
13/09	M	3n	LLR	189
15/09	M	3n	LLR	189
34/09	M	3n	LLR	189
35/09	M	3n	LLR	189
45/09	M	3n	LLR	189
51/09	M	5n	LLLRR	189
52/09	M	3n	LLR	189

Rrid013A

The *lessonae* male possibly produced one L.

The *esculentus* female produced at least one R and possibly an L.

Dosage: No dosage effect detected.

Sample	Sex	Ploidy	Genotype	Loci	
				Rrid013A Al. 1	Al. 2
Father	M	2n	LL	296	
Mother	F	2n	LR	281	296
13/09	M	3n	LLR	281	296
15/09	M	3n	LLR	281	296
34/09	M	3n	LLR	281	296
35/09	M	3n	LLR	281	296
45/09	M	3n	LLR	281	296
51/09	M	5n	LLLRR	281	296
52/09	M	3n	LLR	281	296

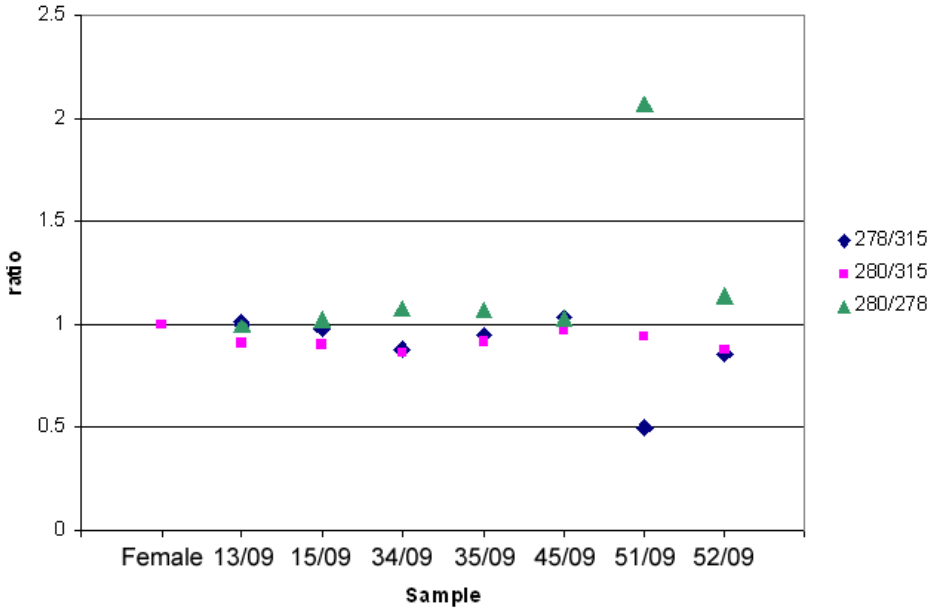
Rrid059A

The *lessonae* male always produced one L.

The *esculentus* female always produced at least one L and one R (diploid gamete).

Dosage: Detectable by the ratio height 280 / height 278 (also detectable in 278/315 and 280/315).

Sample	Sex	Ploidy	Genotype	Expected ratio 280/278	Expected ratio 278/315	Expected ratio 280/315	Loci Rrid059Ared. Al. 1	Al. 2	Al. 3
Father	M	2n	LL	-	-	-	278		
Mother	F	2n	LR	-	-	1	280	315	
13/09	M	3n	LLR	1	1	1	278	280	315
15/09	M	3n	LLR	1	1	1	278	280	315
34/09	M	3n	LLR	1	1	1	278	280	315
35/09	M	3n	LLR	1	1	1	278	280	315
45/09	M	3n	LLR	1	1	1	278	280	315
51/09	M	5n	LLLRR	2	0.5	1	278	280	315
52/09	M	3n	LLR	1	1	1	278	280	315



Re1Caga10

The *lessonae* male possibly produced one L.

The *esculentus* female always produced one R and possibly an L.

Dosage: No dosage effect detected.

Sample	Sex	Ploidy	Genotype	Loci	
				Re1Caga10	
				Al. 1	Al. 2
Father	M	2n	LL	97	
Mother	F	2n	LR	97	135
13/09	M	3n	LLR	97	135
15/09	M	3n	LLR	97	135
34/09	M	3n	LLR	97	135
35/09	M	3n	LLR	97	135
45/09	M	3n	LLR	97	135
51/09	M	5n	LLLRR	97	135
52/09	M	3n	LLR	97	135

RlCA1a27

The *lessonae* male possibly produced one L.

The *esculentus* female possibly produced one L and also one R (no R specific alleles).

Dosage: No dosage effect.

Sample	Sex	Ploidy	Genotype	Loci
				RlCA1a27
				Al. 1
Father	M	2n	LL	111
Mother	F	2n	LR	111
13/09	M	3n	LLR	111
15/09	M	3n	LLR	111
34/09	M	3n	LLR	111
35/09	M	3n	LLR	111
45/09	M	3n	LLR	111
51/09	M	5n	LLLRR	111
52/09	M	3n	LLR	111

RlCA18

The *lessonae* male always produced one L, it shows a mendelian segregation between 179 and 186 (four offspring out of seven inherited allele 179 and three inherited allele 186).

The *esculentus* female always produced at least one L and possibly an R (no R specific alleles).

Dosage: No dosage effect detected.

Sample	Sex	Ploidy	Genotype	Loci	
				RlCA18	
				Al. 1	Al. 2
Father	M	2n	LL	179	186
Mother	F	2n	LR	191	
13/09	M	3n	LLR	179	191
15/09	M	3n	LLR	186	191
34/09	M	3n	LLR	179	191
35/09	M	3n	LLR	186	191
45/09	M	3n	LLR	179	191
51/09	M	5n	LLLRR	186	191
52/09	M	3n	LLR	179	191

Rrid135A

No information on the *lessonae* male gamete production because of L null allele).

The *esculentus* female always produced at least one L and one R (diploid gamete).

Dosage: No dosage effect detected.

Sample	Sex	Ploidy	Genotype	Loci	
				Rrid135A	
				Al. 1	Al. 2
Father	M	2n	LL		
Mother	F	2n	LR	203	236
13/09	M	3n	LLR	203	236
15/09	M	3n	LLR	203	236
34/09	M	3n	LLR	203	236
35/09	M	3n	LLR	203	236
45/09	M	3n	LLR	203	236
51/09	M	5n	LLLRR	203	236
52/09	M	3n	LLR	203	236

Summarization on the 18 loci:

***P. lessonae* male:**

All loci (except Re2Caga3, Res22 and Rrid169A which gave no information because of the absence of L specific alleles) are congruent with the fact that this frog produced haploid L sperms.

For all heterozygote loci (CA1b6, RlCA5, Res20, RlCA2a34, ReGa1a23, and RlCA18) the allele segregates in accordance to Mendel's law of segregation.

***P. esculentus* female:**

All loci presenting an R allele (CA1b6, RlCA1b5, Ga1a19red., Rrid064A, RlCA5, Res16, RlCA2a34, Re2Caga3, Res22, ReGa1a23, Rrid169A, Rrid013A, Rrid059A, and Re1Caga10) show that this frog transmitted at least on R allele.

Loci CA1b6, RlCA5, Res20, RlCA2a34, ReGa1a23, Rrid059A, RlCA18, and Rrid135A show that it also transmitted at least on L allele.

Loci CA1b6, RlCA5, RlCA2a34, ReGa1a23, Rrid059A, and Rrid135A show that it produced L and R alleles at the same time.

This is confirming that the *P. esculentus* female produced at least diploid LR ova.

Dosage effect:

Knowing that all seven studied offspring are at least triploid, loci CA1b6, Ga1a19red., Res16, Res20, RlCA2a34, ReGA1a23 and Rrid059A confirmed, by examination of the relative height of the peaks, that the individual 51/09 is a pentaploid froglet.

Conclusion

All results of the microsatellite study are congruent with the fact that among the seven analyzed offspring six are triploid carrying an LLR genomic constitution while specimen 51/09 is an LLLRR pentaploid.

All triploids are the result of the fusion of a haploid sperm carrying one L genome, with a diploid egg bearing one L and one R genome. The pentaploid specimen has received one L genome from his father and two copies each of the L and R genomes from his mother.

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In memoriam Leszek Berger

The “water frogs’ father” past away the 8th July 2012.

Curriculum Vitae

Personal

Name	PRUVOST
First name	Nicolas Bastien Mathias
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Citizenship	French

Education

2008 – 2013	Dissertation at the Institute of Evolutionary Biology and Environmental Studies, University of Zurich: "Impact of gamete production on breeding systems and population structure of hybridogenetic frogs of the *Pelophylax esculentus* complex", under the supervision of Prof. Dr. H.-U. Reyer
1998 – 1999	Master thesis at National Museum of Natural History, Paris & University Lyon I, France: "Morphometric approach of the European brown frog group (*Rana temporaria*)", under the supervision of Prof. Dr. A. Dubois
1992 – 1998	Study of biology at the University of Paris XII, Paris VI and National Museum of Natural History, France. Main subjects Cellular Biology and Physiology, Ecosystem and Population Biology, Animal and Plant Systematics
1991	Lycée Paul-Valéry, Paris, France, Matura Typus E

Publications

Pruvost, N.B.M., Hollinger, D. and Reyer, H.-U. (2013). Genotype-temperature interactions on larval performance shape population structure in hybridogenetic water frogs (*Pelophylax esculentus* complex). *Functional Ecology* 27: 459-471.

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